Introductory Science Text-Books

PRACTICAL BACTERIOLOGY

AN INTRODUCTION

TO

PRACTICAL BACTERIOLOGY

FOR

Physicians, Chemists and Students

BY

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WITH 9 ILLUSTRATIONS IN THE TEXT, AND 2 PLATES

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PREFACE.

This little work on Practical Bacteriology was written by me to serve as a short text-book for my bacteriological course at the Technical High School, in order to diminish the amount of verbal teaching, and to avoid the continual repetition of necessary directions. The excellent works of Hüppe, Fränkel, and Günther are too voluminous for this purpose, being liable to confuse the beginner from their very fulness of detail. If, however, the student wishes to go more deeply into the subject, he will find these works indispensable.

In consequence, I have, in the following pages, only mentioned the most important methods, and amongst the innumerable ways of staining preparations have only described the most convenient for use. In addition, I have restricted the amount of material to be used as much as possible, so that the subject-matter contained in Chapters I.–XIII., at any rate, should be mastered in one term. The subsequent chapters are meant rather to be used in special individual cases, and on that account those things which are of practical use to the physician or chemist have been treated of

with some detail, whilst other subjects have been only shortly dealt with. Thus the book is also intended to be a short text-book for physicians and chemists in practice, if they wish to occupy themselves in their short periods of leisure with such bacteriological experiments as arise out of their work.

The name *Practical Bacteriology* was suggested by Strasburger's *Practical Botany*, although this work is much more limited in scope.

As to illustrations of apparatus, I have made these as few as possible, in order to keep down the price of the book, having aimed rather at indicating the special form which I consider most suitable than at carefully representing the instruments. This is less important, since all price lists are now so fully illustrated.

On the other hand, I considered it desirable to insert[1] photographs of the most important pathogenic bacteria, because good drawings as a rule are much more distinct than the pictures under the microscope, so that it is frequently enough said by my pupils, "If only the bacteria were as distinctly to be seen as in the picture!" The difference between the microscopical picture and the drawing is often so great, that the beginner can hardly make out the resemblance between them.

W. Migula.

[1] Careful drawings of these photographs have been inserted in place of the photographs themselves.

TRANSLATORS' PREFACE.

In preparing our translation of this little work, which has been made by arrangement with Dr. Migula, we have endeavoured to convey the author's meaning as accurately as possible. In only a few instances has new matter been added, and then invariably in the form of footnotes. These footnotes will be found on pages 7, 21, 105, 212, and 236. In addition the titles of some English works and translations and the names of some English manufacturers have been added in the introduction.

We trust that this book may prove useful to English students; the painstaking way in which the minutest practical details have been described will render it, without doubt, especially serviceable to those who have not had the opportunity of going through a systematic course on Bacteriology.

M. and H. J. Campbell.

London, 1893.

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INTRODUCTION.

Bacteriology is a very wide subject; however, only a small portion of it need be dealt with in a practical course which is not intended for the training of specialists. It frequently happens to both physician and chemist that a few bacteriological experiments are necessary in the course of his ordinary work, and hence that he requires to understand thoroughly the most important methods of manipulation. This book is only intended to supply this need, and the subject is, I hope, treated in a manner which is easily comprehensible by the non-specialist. It only remains to mention the best way of furnishing the laboratory, the places where the necessary appliances can be obtained, and the most important literature on the subject.

Necessary Apparatus.

1. The first and most important instrument is the microscope. If the student intends to devote himself with some assiduity later on to accurate bacteriological work, it is best for him to choose at the outset an in-

strument, to which subsequently it may be possible to add whatever is necessary, as the need arises. In the first place it is absolutely necessary that the stand should be jointed, and that it should be provided with coarse and fine adjustments, with rack and pinion, with a micrometer screw, with an Abbé's condenser and an iris diaphragm, and above all, with as large an object stage as possible. If later on it is desired to take microphotographs, it is best to get an apochromatic lens, as far better results can be obtained by its means. However, these lenses are very dear, and cannot be afforded by every one.

The best microscopes are furnished by Zeiss in Jena,* but they are much more expensive than those manufactured by other firms. Seibert, in Wetzlar, supplies instruments which are very similar to those of Zeiss, and are much cheaper. It would be a good thing if this firm of manufacturers would arrange to have the micrometer screw above as in Zeiss' instruments, as when it is underneath it is inconvenient to use. The following firms also supply good microscopes :—

Paul Wächter, Berlin.
Otto Himmler, Berlin.
A Meyer & Co., Enge-Zurich.

* London Agent, R. Kanthack, 21, Golden Square, Regent Street, W.

E. Hartnack, Potsdam.
C. Reichert, Vienna.
E. Leitz, Wetzlar.
L. Bénèche, Berlin, S.W., Grossbeeren Straasse, 19.
J. Klönne & Müller, Berlin.
F. W. Schieck, Berlin.
Schmidt & Hänsch, Berlin, S.
R. Winkel, Göttingen.
F. Plössl & Co., Vienna.
A. Nachet, Paris, rue St. Severin, 17.

It is sufficient for all ordinary bacteriological work to have a simple microscope, with six different objectives, magnifying about 50, 100, 200, 500, 700, 1,000 diameters; it is also well to have an illuminating apparatus, and, if possible, an oil immersion lens. From my own experience, I can recommend the following as good combinations :—

Zeiss, Stand 6. Illuminating apparatus No. 19, Lenses A, D, and $\frac{1}{12}$ homogeneous immersion. Eye pieces 2, 3, and 4, price £16 2*s*.

Seibert, Stand 4. Small illuminating apparatus. Lenses I., V., and X. Eye-pieces I., II., III., price £16 16*s*. 6*d*.

Other manufacturers supply similar instruments at somewhat lower prices; however, their instruments scarcely reach the excellence of those supplied by the above well-known firms. Unless the

student is himself a judge of microscopes, and so is in a position to discover any defect in them, it is best for him not to choose his instrument himself, but to leave the selection of it to some experienced person.

It will be taken for granted in the following pages that the student understands the use of the microscope, and hence any one who does not possess such knowledge is advised to make himself acquainted with general microscopical technique in some work which treats of this subject; such as—

Frank J. Wethered, *Medical Microscopy*. A guide to the use of the microscope in Medical Practice (Lewis), London, 1892.

Henri van Heurck, *the Microscope, its Construction and Management*, translated by Baxter (Crosby, Lockwood & Son), London, 1893.

Behrens, *Hilfsbuch zur Ausführung Mikroskopischer Arbeiten*. Braunschweig, 1885.

Strasburger, *Practical Botany*, translated by Hillhouse. London (Sonnenschein) and New York (Macmillan), 1888. Third Edition, 1893.

Frey, *Das Mikroskop und die Mikroskopische Technik*. Leipzig, 1886.

2. Slides made of white glass preferably with

ground edges, as they are thus less likely to cut the hand when being cleaned.

3. Hollow slides and slides on to which glass cells have been cemented. The cement ought not to be destroyed or liquefied by heat.

4. Cover-glasses. For ordinary use, 15 mm. in diameter; for cover-glass cultures, however, they should be 22 mm. in diameter; round cover-glasses are preferable, because their edges are not so likely to catch in the cloth when they are being cleaned as the square ones, and because they are more suitable for permanent preparations.

5. Watch-glasses of about 5 cm. in diameter, with flat bottoms, for holding the staining fluids, etc. Embryo cups, which consist of blocks of compressed glass, in which depressions have been ground, are very useful, only they are rather dear.

6. Glass rods, thin and thick. It is best to fuse the platinum wire into the latter, as it is apt to become loosened from the thin rod on cooling.

7. Glass tubing of various sizes, for various purposes; amongst others, for making cultivations of anaërobic bacteria.

8. Flasks. A few large ones for preparing nutrient gelatine, etc., and smaller ones for cultivations.

9. Small Erlenmeyer's flasks, very useful for bouillon cultures, etc.

10. Glass funnels; large for filtering the nutrient

media, and small for filtering staining solutions. A few quite small ones, about 2 cm. in diameter, are also required for filtering the staining solution direct upon the cover-glass.

11. Small glass benches, with their ends bent over, upon which to stand cultures (Fig. 8).

12. Test tubes, of at least 15 mm. in diameter and 15 cm. in length. For anaërobic cultivations longer ones, and for Esmarch's tubes wider ones are required. A considerable number of tubes are needed, and in procuring them it is unwise to be too economical.

13. Beakers. These are needed for various purposes; I myself use them for boiling the nutrient gelatine. It is also convenient to sterilise small glass tubes, pipettes, etc., in beakers in the hot-air sterilising apparatus.

14. A wash-bottle of about 250 grms. capacity, with a finely drawn out tube.

15. Two graduated measures for measuring out the quantity of water in preparing the nutrient media—one of 1 litre in capacity divided into ten parts, and another of 100 cm. divided into twenty parts.

16. Measuring pipettes, several of 1 ccm. in capacity divided into tenths, and others of 2, 5, 10, 20, 25, 50, and 100 ccm. capacity.

17. Glass dishes with double covers, for the so-called plate cultures, of about 10 cm. diameter; the lids should fit as well as possible.

18. Glass shades with glass knobs, of about 22–24 cm. in diameter, and 5–6 cm. in height. These are made use of as moist chambers for potato cultivations, etc. (Fig. 1).

19. Porcelain dishes of about 5 cm. in diameter for heating staining solutions, and one larger one which is used for heating the nutrient agar-agar in the salt water bath.

20. A Kipp's apparatus for the preparation of hydrogen for anaërobic cultivations is not indispensable. In case of need, hydrogen can be prepared in a simple gas generating flask, although of course the operations cannot be as accurately carried on as if a Kipp's apparatus is used. A small apparatus costs about 10*s.*, and is to be strongly recommended.

21. Thermometer. This need only indicate up to 150° C. A thermometer with a long stem divided into tenths of degrees, indicating up to 50° C., is very convenient for use in the incubator.

22. A Reichert's thermo-regulator* is necessary for cultivations of tubercle bacilli, if gas is used, and if the incubator is good.

23. Gas burners with one and four flames, the latter for the hot air steriliser and the steam steriliser. The ones most to be recommended are those of Desaga

* Page's thermo-regulator is, in some respects, more convenient.

(Heidelberg), which have an arrangement for regulating simultaneously the supplies of gas and air.

24. A micro-burner for warming the incubator. Koch's safety burners are the best for this purpose, but they are unreasonably dear. See page 89.

25. An incubator. However, unless great uniformity of temperature is necessary, this can be replaced by the very simple apparatus described on p. 83.

26. A steam steriliser (Fig. 4).

27. A hot air steriliser (Fig. 3).

28. A balance. Should a delicate chemical balance be unobtainable, an ordinary pair of scales for weighing gelatine, peptone, staining reagents, etc., will answer the purpose. Great exactness is only essential in a few cases; for example, in the preparation of normal solutions, such as the caustic soda solution used for staining flagella. In such cases, however, the reagents can always be obtained ready-made from a reliable chemical manufacturer, or they may be ordered at a chemist's or from a chemical laboratory. The need for a delicate balance so seldom arises that it is hardly worth while to procure one.

29. A filter stand.

30. Filter paper, somewhat thick for filtering gelatine and agar-agar, and a thinner kind, not very permeable, for filtering the staining solutions. Folded filter papers are used for gelatine: these can be obtained ready-made. For staining reagents it is best

to use the circular filter papers, which are prepared with hydrochloric and fluoric acid. In addition, some soft absorbent blotting paper is constantly required for taking up excess of moisture from slides, cover-glasses, etc.

31. Litmus paper, blue and red. This is absorbent paper, coloured with litmus solution; a drop of the solution to be tested is allowed to fall upon it from a glass rod. The more sensitive the litmus paper is, the more useful is it, but of course the more difficult is it also to work with. The blue and red papers should be kept separate, and should be protected from the light, preferably in large bottles with smooth stoppers, which have been smeared over with vaseline, the bottles themselves having been blackened with varnish.

32. Cotton wool. Clean cotton wool free from grease, such as can be bought from pharmaceutical chemists.

33. India-rubber stoppers and caps for test-tubes and india-rubber tubing. Some of the stoppers should be perforated.

34. Platinum wire of about ·8–1 mm. in thickness. For most purposes only short lengths of the wire are needed; but as long pieces are required, as for anaërobic cultivations, it is as well to have it in pieces about 12 cm. long. A few mm. of the wire are fused on to the ends of thick glass rods; some

of these should have their free ends filed into smooth, short points, and others should have them bent over to form small, closed, oval loops, of about 2 mm. in their greatest diameter.

35. Forceps, which must not have too strong a spring.

36. Needles. The most convenient arrangement is to have nickel-plated metal needle-holders, in which ordinary sewing needles of different sizes can be fixed: as this apparatus is convenient to sterilize.

37. Scalpels. One should be of platinum; it may be prepared in the manner described in a note on p. 123.

38. Razors and strops. It is important that the razor should have a broad blade, and should be flat upon one side. The strop should be made of tightly stretched leather. A glued leather strop is not to be recommended.

39. Various bottles. For alcoholic staining solutions the most convenient kind of bottle is one provided with a cork through which a glass tube with a drawn out lower end passes. The tube should be long enough to reach the bottom of the bottle, and it also ought to fit loosely in the cork, so that it can be easily drawn out. Then when liquid is wanted for use the tube is drawn out of the bottle, the upper end being closed with the finger. By this means a sufficiently small quantity of the staining solution is ob-

tained. As to the other kinds of bottles which are needed, it will be seen later what is required.

40. Several short glass tubes supplied with well fitting india-rubber caps at one end, the other end being drawn out so as only to leave narrow openings. In the absence of ready-made india-rubber caps, one can use pieces of thin india-rubber tubing tied up tightly at one end. These latter are very useful for taking up staining solutions and dropping them upon the cover-glasses.

41. Distilled water in several bottles of one litre capacity.

42. Absolute alcohol.

43. Xylol.

44. Canada balsam.

45. Cedar-wood oil.

46. Aniline oil.

47. Sulphuric acid.

48. Nitric acid.

49. Hydrochloric acid.

50. Acetic acid.

51. Glycerine.

52. Concentrated solution of soda.

53. Sodic hydrate.

54. Potassic hydrate.

55. Potassic iodide.

56. Paraffin.

57. Vaseline.

58. Iodine.
59. Carbolic acid.
60. Corrosive sublimate.
61. Gelatine.
62. Agar-agar.
63. Peptone.
64. Liebig's extract of meat.
65. Ordinary salt.
66. Grape sugar.
67. Fuchsine.
68. Methylene blue.
69. Methyl violet or gentian violet.
70. Bismarck brown.
71. Various horn spoons, spatulæ, ordinary knives, cloths for wiping, etc.
72. Wolfhügel's counting apparatus (Fig. 9).

The following firms may be recommended:—

For Bacteriological Apparatus.

F. & M. Lautenschläger, Berlin.*

F. E. Becker & Co., 33, Hatton Wall, Hatton Garden, London, E.C.

A. Gallenkamp & Co., 6, Cross St., Finsbury, London, E.C.

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* London agent, Kanthack.

Dr. Robert Muencke, Berlin.
Dr. Hermann Rohrbeck, Berlin.
Klönne & Müller, Berlin.

For Microscopical Appliances.

Stanley, Railway Approach, London Bridge, S.E.
R. & J. Beck, 68, Cornhill, London, E.C.
Theodor Schröter, Leipzig.
Alt, Eberhardt & Jäger, Ilmenau, Thüringia.
Heinrich Böcker, Wetzlar.
R. Jung, Heidelberg.
Klönne & Müller, Berlin.
Dr. G. Schreiber, Chemnitz i. S.
Dr. Bender & Hobein, Münich.
Marpmann & Schurig, Leipzig.
Adolph Veit, Heidelberg.
Fritz Fischer & Röwer, Stützerbach, Thüringia.

For Chemical and Staining Reagents.

Baird & Tatlock, 14, Cross St., Hatton Garden, London, E.C.

Hopkin & Williams, 16, Cross St., Hatton Garden, London, E.C.

Townson & Mercer, 89, Bishopsgate St. Within, London, E.C.

E. Merck, Darmstadt.

Dr. Grübler, Leipzig.
Klönne & Müller, Berlin.
Dr. G. Schreiber, Chemnitz.
Th. Schuchhardt, Görlitz.
Trommsdorf, Erfurt.
Dr. G. Münder, Göttingen.
Heinrich Sohncke, Halle.
Dr. K. Roth, Berlin N., Strassburgerstr. 18.

For Microscopical Preparations.

Klönne & Müller, Berlin.
Heinrich Böcker, Wetzlar.

For Apparatus for Microphotography.

Otto Perutz, Münich.
Albert Glock, Karlsruhe i. B.
Schippang & Wehenkel, Berlin.

Dr. Burstert and Fürstenberg make excellent micro-photographs of preparations which are sent to them. In addition, the author has found their glass plates very convenient, especially for demonstrating.

More Detailed Works on Practical Bacteriology.

Hüppe, *Die Methoden der Bacterien-Forschung*, 5te Auflage. Wiesbaden, 1891.

Carl Fränkel, *Grundriss der Bacterienkunde*, 3te Auflage. Berlin, 1890.

Carl Günther, *Einführung in das Studium der Bacteriologie.* 2te Auflage, 1891.

R. Wurtz, *Technique Bactériologique, Encyclopédie Scientifique des Aide-mémoire* (Gauthier-Villars et fils), Paris, 1892.

Crookshank, *Manual of Bacteriology* (Lewis), London.

George M. Steenberg, *A Manual of Bacteriology*, New York (Wood & Co.), 1892.

Important Works on Bacteriology.

Flügge, *Micro-organisms*, trans. by Watson Cheyne, M.B., New Sydenham Soc., London, 1890.

Eisenberg, *Bacteriological Diagnosis*, trans. by Norval H. Pierce, M.D., Philadelphia and London.

Baumgarten, *Pathologische Mykologie.* Braunschweig, 1890.

Baumgarten, *Jahresbericht über die Fortschritte in der Lehre von den pathogenen Mikro-organismen*, pub. annually since 1886.

Centralblatt für Bacteriologie und Parasitenkunde, pub. since 1887.

Annales de l'institut Pasteur, Paris.

All additional literature on the subject is mentioned in these works, which are quite indispensable to any student who wishes to study Bacteriology thoroughly.

Pure cultures can be obtained from Lautenschläger, Klönne und Müller, or from Krall, (II. Opatowitzer Gasse, 9, Prague, Austria.)

CHAPTER I.

EXAMINATION OF LIVING BACTERIA.—THEIR STRUCTURE AND FORM.—HOW TO OBTAIN MATERIAL FOR EXAMINATION.

Bacteria are distributed everywhere in nature; they cling to the surface of every substance; they are to be found in fewer or greater numbers in dust, in water, and in air. We only perceive their presence under ordinary circumstances, however, when they find conditions favourable for their rapid growth and development. Generally they are detected by the odour they produce; sometimes, too, they impart a colour to the substance upon which they are growing, or sometimes they themselves acquire a coloration of their own, when they are spread over the objects in thick, more or less slimy masses. Although it is easy enough to find bacteria in such masses, yet it is both simpler and more convenient to grow the species to be examined artificially, especially as this can be very easily accomplished in several different ways. Moreover, different forms are obtained, according to the method adopted; and this, of course, is very useful in the study of bacteria.

If certain plants, preferably some green Algæ, such as Spirogyra, Vaucheria, or Cladophora, are left for a short time in a small quantity of water, so that they become decomposed, one very soon perceives, by the unpleasant odour produced, that bacteria have begun their destructive processes. The water becomes turbid, and the sides and bottom of the vessel become covered with a slimy film, which grows thicker and thicker, till at last here and there small flakes become detached from the rest. Sooner or later, according to the temperature, the surface becomes covered with a dirty white skin, which at first is very delicate, but which frequently, later on, becomes of a gelatinous consistency. The turbidity of the water, the slimy covering of the vessel's walls, and the skin on the surface (the bacterium skin), all indicate the presence of bacteria, which have multiplied in enormous quantities at the expense of the dying Algæ. The bacteria were originally present in proportionately a small quantity, both in the water and attached to the Algæ themselves and to the walls of the vessel.

Similarly bacteria can easily be obtained for examination by cutting up carrots or potatoes, boiling them, and allowing the resulting liquid to stand in broad open vessels. Essentially the same appearances manifest themselves as with the decomposing Algæ, only as a rule not nearly so many species are obtained.

Generally quite different ones make their appearance when finely pounded meat is mixed with ditch-water, and left to decompose.

In all these ways a mixture of bacteria is obtained; that is to say, bacteria of very different forms and kinds are found mixed together. It is, moreover, impossible to say beforehand which forms will be developed, for this depends on the kind of germs which happen to be present, and also on the varying chemical constitution of the special nutrient fluids prepared as above.

By the following method, a pure culture—that is to say, one in which only one kind of bacterium develops itself—may be obtained. Hay is left in water for twenty-four hours; the resultant liquid is then strained through a linen cloth, and diluted, until a volume, ten centimetres in depth, is of a light chestnut-brown colour.

The success of this experiment depends upon the fact that the spores of the hay-bacillus (*Bacillus subtilis Ehrenberg*) can endure the heat of boiling water for an hour without being killed. The liquid is poured into a Florence flask, until the latter is three-quarters full. The mouth of the flask is then stopped up with a cotton-wool plug, and its contents are kept simmering gently—only a small amount of steam being formed,—for an hour. In this manner all other organisms and their germs are killed, the spores of the hay-

bacillus alone being able to withstand the heat. These now begin to develop in a most vigorous fashion, for the hay-infusion, which contains the spores, is in itself a most favourable nutrient medium for the hay-bacillus. As all other organisms have been killed, and the cotton-wool stopper prevents the entrance of new ones, a pure culture of the hay-bacillus is obtained in this manner. To be sure, there is no longer any doubt that, under the name of hay-bacillus, there are included many different kinds of bacteria, which for the most part are very closely allied. This, however, for the present, need not be considered, as in the following experiment we shall occupy ourselves with the form and development of the different kinds, rather than with their rigid classification, which will be treated at some length in later chapters.

Finally a comparatively pure original material for bacteriological experiments may be obtained in the following manner:—Slices about one centimetre thick of cooked turnips and potatoes are placed for a time in different places—in the open air, in a cellar, on the ground, or in a sitting-room. They are left in these places for two or three hours freely in contact with the air, and are then further cultivated in moist chambers. The moist chamber (Fig. 1), which has been successfully used up to now, consists of two glass vessels, the upper and smaller one, which is supplied with a handle, fitting closely into the under one. Before use, these

vessels should be carefully cleansed. This is most successfully accomplished with a solution of corrosive sublimate, 1 : 1000 (it is advisable to have a solution of this strength always handy, as it is in constant use).[1] A piece of filter-paper, soaked in this solution, is then placed at the bottom of the dish, and the piece of turnip or potato is placed upon it, the same side as before being uppermost. The upper glass is then put over, and the vessel is kept at the temperature of an ordinary sitting-room. After a few days

Fig. 1.

small variously shaped masses appear on the slice in the form of little droplets, dry flat scales, or more

[1] In the use of corrosive sublimate as a sterilising agent, it is of importance to remember not to use more of the solution than is absolutely necessary, lest so much of the salt should be left adhering to the sides of the dish, after the water has evaporated, that it may fall on the culture and kill the bacteria. It is further necessary to bear in mind the fact, that, if albuminous substances come into contact with the corrosive sublimate solution, the mercury is precipitated, and thus the sterilising properties of the solution are more or less destroyed. For these reasons it is advisable to sterilise by means of heat whenever possible.

or less slimy masses with irregular outlines. These masses are generally whitish or yellowish, being rarely of any other colour. They are colonies of bacteria, which, if they are situated sufficiently far apart from each other, present small pure cultures of the different species. While the slices of turnip were lying in contact with the air, bacteria germs fell upon them. These, if the soil suits them, develop at the spots, where they fall, into small colonies, which remain isolated upon the firm medium, and only can become mixed with other bacteria, if neighbouring colonies, in consequence of their continued growth, touch each other.

Occasionally, however, it may happen that two germs of different kinds fall upon the same spot, and that thus the resulting colony is impure from the outset. As a rule, however, it may be taken for granted, that colonies which are pure at the beginning are obtained by this method, and these afford us the original material for the pure cultures to be described later. Generally colonies of moulds (especially of the Penicillium glaucum, or of species of the genera Aspergillus and Mucor) develop amongst the bacteria colonies; these are easily to be recognised, as it can be readily seen with the magnifying glass, or indeed with the naked eye, that they are composed of long threadlike hyphæ. Later on, these hyphæ raise themselves above the surface, and develop reproductive organs.

by means of which they can immediately be distinguished from the others.

If we now take with a needle a small portion of such a bacterium colony from the surface of a potato, and spread it out in a drop of water upon a slide, cover it with a cover-glass, and examine it with a magnifying power of about 500 diameters, and with a small opening in the diaphragm, we shall find in most cases a specimen of the genus *Micrococcus*, or more rarely, a rod-bacterium. The Micrococci are small round cells, which on an average are $\frac{1}{1000}$ mm. in diameter; sometimes they are a little larger, sometimes a little smaller, according to the species which happens to be present. They are almost always motionless. Up to now we know very few kinds of Micrococci which, like the *Micrococcus agilis Cohn*, move of their own accord. The trembling movements exhibited by most Micrococci are not manifestations of their own activity, for they are also exhibited by non-living bodies suspended in fluid, such as small particles of carmine. This phenomenon has not yet been fully explained, but apparently it is caused by the neutralization of the electrical tension of very small bodies. The peculiarity of these so-called *Brownian movements* consists in this, that the particles, in spite of their continual trembling and dancing, never move from place to place, but remain practically at one point.

Presently, however, very disturbing movements are

to be seen. The water at the edge of the cover-glass evaporates, and thus different currents are continually being created. The bacteria, which are in the field of vision, are swept along by these currents, and thus the difficulties of observation are much increased. In consequence the following plan has been successfully adopted for such observations :—A slide is chosen, in the middle of which either a round hollow or depression has been ground, or better still, on to which a glass ring has been cemented. A small drop of water is now placed on a cover-glass, and the bacteria are spread out in it; the cover-glass is then turned over and placed over the depression in the slide, so that the drop of water hangs freely in the hollow space, and nowhere touches the bottom or the sides. A small drop of olive-oil is then placed at the edge of the cover glass, and this spreads itself out in a circle between it and the slide, making the little hollow quite air-tight. The drop of water is in this manner prevented from evaporating, and in addition is protected from the entrance of new germs from the outside, which, in later experiments, is of great importance. In this *moist chamber* the development of the bacteria can be very well observed; and this method of culture, when the drop of water has added to it a drop of nutrient fluid, is called *cultivation in the hanging drop*.

If some material from another colony, different perhaps in form or colour, be now placed in such a hang-

ing drop, it is possible that roundish cells may again be found, which even with the microscope can hardly be distinguished from the first ones. But the naked-eye appearance of the colonies was quite different, the first being perhaps white, and the second yellow. We observe, therefore, that these characteristics of form are not sufficient in themselves to distinguish the different species from one another, and that, in addition to the morphological characteristics, we must take the culture characteristics into account. A third colony may have the same yellow colour as the second, whilst appearing somewhat drier. If we spread out a small portion of it in the hanging drop, we find with the microscope quite another effect. The single cells may be roundish again, but they cling together in eights, or in multiples of eight, forming compact ball-like little bundles. We should then have a specimen of the species Sarcina (wool-pack fungus), which belongs to the group Cocci, or ball-bacteria, but which forms a genus by itself. We are not likely to come across a third genus, *Merismopedia*, as it is comparatively rarely met with. In this genus the cells join together in packets of four, or of a multiple of four; but the packets are not, as in Sarcina, several layers thick, but only one layer thick. This genus is also called Merista.

On the other hand, we are very likely to find several different varieties of the genus Micrococcus. If the

little round cells are grouped into thick irregular heaps, they are called *Staphylococci*; if they form chains, like strings of pearls, *Streptococci*; and if they lie together in pairs, *Diplococci*. All possible sorts of intermediate forms are met with, varying according to their nutriment, or to other similar conditions.

Quite a different appearance is presented if a drop of putrid-meat infusion be taken up with a loop of platinum wire, and examined in the hanging drop. It is seen to be swarming with active, lively organisms of very various kinds. There are chiefly elongated cylindrical cells, "rod-bacteria," which are sometimes scarcely longer than they are broad, but at others twice, or several times as long, and which occur in all intermediate gradations. There are also great differences in their thickness; generally they are about $\frac{1}{1000}$ mm. thick, but they may be either thicker or thinner. Further, we frequently find two or more cells united together to form a chain or thread, being sometimes loosely and at others quite closely connected. The rod-bacteria are divided into two classes, *Bacillus* and *Bacterium*; it is, however, impossible to draw a sharp line of distinction between them. Generally under *Bacillus* are included those forms which are more extended, which **form resting spores**, and which, as a rule, are motile: and under *Bacterium*, those which are shorter, which **form no spores**, and which possess no power of

locomotion. Moreover, it very frequently happens that these characteristics are not all present in one species; many are motionless, and yet form spores, and so on. Continually species are being taken out of the class *Bacterium*, to be included in the class *Bacillus*, and in the end the former will probably be quite absorbed in the latter.

We soon observe considerable differences in the movements of bacilli. Some shoot like lightning through the field of vision, whilst others drag themselves slowly forwards. Again, others show oscillatory movements without really moving from one place, whilst yet others swarm about amongst themselves without apparent method, now shooting here, now there, sometimes pausing for a moment, and then turning round, etc. Many bacilli are quite motionless, whilst in others these movements may be temporarily suspended.

Distributed amongst these rod-bacteria, we find the motionless ball-bacteria, or Micrococci, which are already known to us. Specimens of a third family, the **Screw** or **Spiral bacteria**, may occasionally be found in the meat infusion, but they more frequently occur in an infusion of decomposing plants. They are like little bent rods, which sometimes appear as long threads, twisted into the form of spirals, and sometimes as shorter cells, which present the appearance of half the turn of a spiral. The diameter of the

spiral varies considerably, as does also the thickness of the cell itself. Some of these Spiro-bacteria are exceedingly motile, others are rather inert, whilst yet others are completely motionless. They have been divided into three classes, *Spirochæte*, *Spirillum*, and *Vibrio*. There is, however, no sharp boundary line to be drawn between these classes, as nearly every investigator includes different species under different headings. We shall study the classification of bacteria more fully in the chapter entitled "**Formation of Spores.**"

A fourth family of bacteria, the **Thread-bacteria**, or **Desmo-bacteria**, is not likely to make its appearance. The three most important classes of this family are *Cladrothrix*, *Crenothrix*, and *Beggiota*. The Beggiota form long, motile threads, consisting generally of colourless cells, and are distinguished by the presence in their cells of numerous strongly refractive granules, which probably consist of sulphur. They occur in sulphur springs, and in dirty water that is rich in decomposing albuminous bodies.

The class *Crenothrix* forms simple threads, the separate cells of which surround themselves with a distinct sheath, and then change themselves by segmentation at their ends into small roundish spores. The threads are motionless, and especially in their younger stages group themselves together into little patches. They are especially frequently found in

Fig. 2.

I. Coccaceæ, Ball-bacteria. 1. Staphylococcus. 2. Diplococcus. 3. Streptococcus. 4. Merismopedia. 5. Sarcina.

II. Bacteriaceæ, Rod-bacteria. 1. Anthrax-bacillus. 2. Typhoid-bacillus. 3 and 4. Various bacilli obtained from water.

III. Spiro-bacteria, Spiral-bacteria. 1. Comma form of Cholera-spirillum. 2. Spiral form of Cholera-spirillum. 3. Very thick. 4. Very thin and irregular Spirilla from foul mud.

Magn. 1000. The heavily leaded bacteria are copied from coloured preparations.

North Germany, and have caused real calamities by stopping up the water pipes.

Finally, the third class, *Cladothrix*, also forms threads, which possess pseudo-branches; the separate cells are also enclosed in sheaths; they are generally found in dirty water, in refuse water from factories, etc.

Besides the classes already mentioned, a number of others have been defined; but as some of these only very rarely occur, and the others may be included under those already mentioned, we need not consider them here.

After having in this manner learnt to recognise superficially the forms of various bacteria, it is easy to classify them, according to their forms, into four large classes:—

1. Coccaceæ, Ball-bacteria. Cells spherical, or shortly elliptical. (Fig. 2, I.)

2. Bacteriaceæ, Rod-bacteria. Cells distinctly longer than broad, straight rods, varying in length. (Fig. 2, II.)

3. Spiro-bacteria (Vibrionaceæ), Corkscrew-bacteria. Cells curved, often twisted into long or short spirals. (Fig. 2, III.)

4. Desmo-bacteria, Thread-bacteria. Cells united to form long threads, some being enclosed in sheaths.

This system of classification, originated by Ferdinand Cohn, is the easiest to use, being the one by

means of which a specimen may be most quickly classified; whilst other systems, although perhaps more scientifically correct, are much more difficult to use, and on that account would not serve our present purpose.

As concerns the more minute structure of the bacterium cell, there is not much to be said; a differentiation in its interior is only very rarely to be observed, even in the largest forms. Only so much is certain, that all bacteria possess, in addition to their protoplasm, a membrane which, as it appears, is generally of an albuminous constitution, but more rarely consists of the same materials as that of ordinary plant cells, namely cellulose.

So far no nucleus has been demonstrated in bacteria, but lately it has been considered that the whole contents of the cell is composed of nuclear substance.

CHAPTER II.

PREPARATION OF NUTRIENT MEDIA.

In the cultures of bacteria, described in the preceding chapter, the store of nutrient material necessary to them becomes gradually used up by the activity of these organisms, and little by little development and reproduction comes to a standstill. Some of the bacteria die from want of nourishment; whilst others, in a manner to be considered later, form permanent forms, or "spores," which are able to remain quiescent for a long time until favourable conditions for growth reappear. In order to continue the propagation of bacteria, it is necessary to convey them from time to time into new nutrient media. Of course a substance similar to that in which the bacteria were first cultivated is suitable for their further cultivation; nevertheless, as a rule, other substances are chosen, which possess in many respects considerable advantages over the former ones. These substances are universally designated as nutrient media, and are divided into solid and fluid media. The preparation of these is not difficult, but it requires great care and accuracy in carrying out directions in order to ensure success.

Amongst fluid media we need only consider milk and *bouillon*. The latter is most successfully prepared in the following way :—Half a kilogramme of pounded or minced beef, as free as possible from fat, is well stirred up in a litre of water. The mixture is allowed to stand for from twelve to twenty-four hours (if the weather is warm it is placed on ice). It is then squeezed in a strong gauze bag, until about one litre of liquid is pressed through. Ten grains of dry peptone and five grains of common salt are added to this reddish fluid, which is neutralised by being boiled in a large beaker in the steam steriliser with a saturated solution of chemically pure soda (sodium carbonate) ; or better still, the fluid is made slightly alkaline, and is then boiled for an hour in the steam steriliser. By this means all the albumen, which coagulates at boiling point, is separated out, and a clear light yellow fluid, which no longer becomes turbid on being boiled, is obtained by filtering.[1] This filtering must take place whilst the fluid is hot ; if it should, however, happen that the filtrate is not quite clear, it must be allowed to cool ; the white of a new-laid egg

[1] If the glass vessels, in which the bouillon is later on sterilised, have not been used before, and especially if they have not been boiled, the liquid is often rendered turbid, because the glass has an alkaline reaction. It is therefore necessary to boil all vessels before using them, if faultless preparations are desired.

is then added to it, well mixed with it, and the mixture boiled for a few minutes. The coagulated albumen sinks to the bottom of the beaker, carrying with it all the small impurities which were suspended in the fluid, and another filtering is sure to yield a clear preparation.

The milk is placed at once in the vessels, to be used for the cultivations of the bacteria, and is boiled in them.

Amongst the solid nutrient media we shall consider the following: nutrient gelatine, nutrient agar-agar, blood serum, and potatoes.

Nutrient gelatine can be prepared in various ways, and may have various compositions. The kind which is best adapted for most purposes is obtained in the following manner:—The meat-juice is prepared in the same way as for the nutrient bouillon, but 50 to 100 grammes of the finest white gelatine are added to it, as well as the ten grammes of peptone and five grammes of common salt. Ordinary commercial gelatine is generally not white enough; if it is used for the preparations, it imparts to them a darkish coloration, which is a great drawback in certain experiments. The gelatine, peptone, and salt are dissolved in the warm liquid by constant stirring; the liquid is then boiled up, neutralised in the manner described above, boiled for half an hour, and then filtered. In order to keep the liquid hot, whilst it is being filtered, the hot-water

funnels, which were formerly so much recommended, may be used. It is however much better to place the whole filtering apparatus in the steam steriliser, keeping the temperature at between 60° and 80° Centigrade, in order to prevent the solidification of the gelatine. This can be done by putting the funnel containing the liquid nutrient gelatine into a sufficiently large Florence flask, and placing both in the steam steriliser. In order to prevent the steam, which condenses on the walls of the funnel, from becoming mixed with the gelatine, a thick ring of cotton wool is placed in the mouth of the flask round the stem of the funnel. The gelatine does not always take the same time to pass through the filter; sometimes the whole litre will be filtered in a quarter of an hour, sometimes one must wait for hours. As before, the filtrate is occasionally turbid; in this case the liquid is cooled to 10° C., and the white of an egg is well stirred into it; the mixture is then boiled up and refiltered.

In order to prepare nutrient agar-agar, twenty grammes of agar-agar are added instead of the gelatine. Agar-agar is a vegetable substance, procured from marine Algæ; it is generally obtained commercially in the form of long threads, which must be pounded up as finely as possible, in order to render them easily soluble. If the threads are only cut up into little bits, it takes much longer to dissolve them completely. The composition of nutrient agar-agar is as follows:

one litre of meat juice, twenty grammes of agar-agar, ten grammes of peptone, five grammes of common salt. The mixture is boiled in the steam steriliser until the agar-agar commences to dissolve; this takes about four hours. Since the agar-agar only becomes completely dissolved at a higher temperature than the boiling point of water, and further, since the boiling point of the solution rises above 100° C., as it becomes stronger, the operation is now best carried on in a bath of concentrated salt solution. The agar-agar solution is in this manner kept boiling from two to three hours, until it becomes quite liquid and homogeneous. It is now filtered, being kept at boiling point in the steam steriliser. This filtering is a somewhat wearisome process, which can only be shortened by the thorough boiling of the solution in the salt-water bath. Even then it takes many hours, or even days, before all has passed through the filter. When agar-agar is liquid, it is quite transparent, and of a somewhat darker yellow than gelatine; when, however, it solidifies, it is only transparent in thin plates; somewhat thicker plates are only translucent, and in a mass it is nearly opaque. Should the liquid be not quite clear after filtration, it must, like the other solutions, be cleared with the white of an egg, in order to procure a nutrient medium suitable for all purposes. In order to avoid this wearisome business of filtering, many bacteriologists pour the solution into a tall

cylinder, which they keep standing for a long time in a warm chamber at a temperature of 80°, until the impurities have settled down at the bottom. The clear liquid is then drawn off with a pipette. By this means a nutrient medium quite suitable for many purposes is obtained; but the beginner is strongly advised not to try this method, as the preparation is never absolutely perfect, and at first he must never be satisfied with less. Delicate little flakes are always present in the solution, and these are sure to find their way into the culture tubes, and to create difficulties in various ways. An unpractised student is often unable to distinguish such impurities from the bacteria present in the medium. They may also completely hide from him colonies of bacteria, which have either entered accidentally, or which have remained in the medium in consequence of imperfect sterilisation, and finally they may considerably alter the appearance of an artificially prepared culture.

As such very great care is necessary to prepare nutrient agar-agar, it is best only to use it in such cases, later on to be enumerated, for which nutrient gelatine is not suitable as a nutrient medium. The same is true of blood serum, which can be procured, already sterilised and fit for use, from several different firms. This is advisable, as the preparation of it is so very difficult, that the beginner almost always makes a failure of it. Fluid blood serum is obtained

in the following way:—It is prepared from the blood of animals (oxen or sheep) at the slaughter-house. First the skin, and the instruments to be used for the opening of the blood vessels, are freed from germs, or sterilised, in a manner to be described later. An artery is then opened and the blood is received directly into sterilised vessels, which are covered up and allowed to stand in as cool a place as possible until the red blood corpuscles have separated themselves from the fluid plasma; this plasma is then drawn off with a pipette, which has been sterilised beforehand by being strongly heated.

It is best to put the fluid blood serum straight into the vessels which are to be used for the cultivations, that is to say for the most part into test-tubes. These, however, must be most carefully sterilised beforehand, by keeping them for two or three hours at a temperature of 160° C. This is done in the following manner:—The test-tubes are carefully cleansed, and are fitted with tight-fitting plugs of cotton wool. They are then placed upright in a wire basket, which is kept for some hours in the hot-air steriliser at a temperature of from 140° to 160° C.

The hot-air steriliser (Fig. 3) is a box of sheet-iron, the walls of which are covered with asbestos, in order as much as possible to prevent the loss of heat. It is best for the box to have double walls, between which there is a non-conducting stratum of air. On the top

of the box there are openings to admit a thermometer and a thermo-regulator, which are passed through tight-fitting corks. The apparatus is now heated with Bunsen burners, two, three, or four, according to the size of the box, until the required temperature is

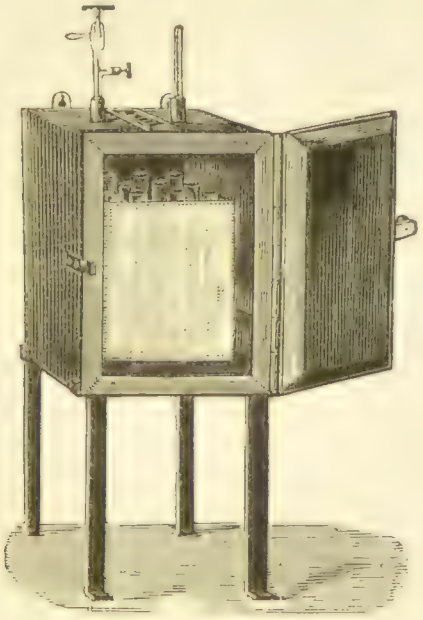

Fig. 3.

obtained. If gas is not available, a smaller apparatus may be heated with specially constructed petroleum lamps.

The test-tubes and cotton-wool are completely sterilised if they are kept in this apparatus for three hours at a temperature of 140°–160° C. This method is called

sterilisation with dry heat; it is especially useful for killing bacteria on instruments and in apparatus, which are not injured by being subjected to great heat for a long time.

The blood serum is taken up with a pipette, which has been sterilised in a similar manner, and is poured into these sterilised test-tubes, until they are a third-part full. The cotton-wool plugs are then immediately replaced. The best method of manipulation is as follows:—The cotton-wool plug is held between the third and fourth fingers of the right hand, whilst the empty test-tube is held in the left. The sterilised pipette is held by its upper end between the thumb and middle finger of the right hand; the blood serum is sucked up, and the upper opening is stopped up with the index finger. The fluid is now allowed to flow into the empty test-tube, after which the latter is immediately closed with the cotton-wool plug. The test-tube is then replaced, a second one is taken with the left hand, the stopper withdrawn as described above, etc., until they are all filled. A word of caution is necessary. The greatest possible care must be taken *not to touch any unsterilised object with the pipette whilst the test-tubes are being changed, nor to lay it down*, as otherwise there is considerable danger of introducing bacteria into the serum, and thus rendering it useless. The same is true of the cotton-wool stopper, which is held whilst the test-tube is being

filled. If by accident the pipette touches any object, or if inadvertently it is laid down, it must be set aside for the time, and another sterilised pipette must be taken. Above all, everything must be done as quickly and skilfully as possible, the operator continually bearing in mind that to the surface of all unsterilised objects innumerable bacteria are clinging, which the slightest disturbance may shake on to the sterilised object, thus rendering it unfit for use. In consequence, it is absolutely necessary to thoroughly wash one's hands in corrosive sublimate solution (1 : 1000), in order to free them from all germs, before filling the test-tubes.

The test-tubes containing the blood serum may be now subjected to "*fractional or intermittent sterilisation*," by exposing them for an hour a day for eight days to a temperature of 58° C. This is most conveniently accomplished in an incubator, which is described later, and in which the temperature can be very accurately regulated and kept constant. The blood serum may be sterilised in a similar manner after it has solidified; but this is not to be recommended, as it loses in transparency and also parts with a great deal of the moisture it contains during the operation. This method is not really certain to free the blood serum from germs, as there are bacteria which, even in the vegetative condition, are quite able to withstand this temperature. Although a relatively successful result

is obtained, the only really safe method is to employ the greatest care in taking the blood serum, since the blood of healthy animals is free from bacteria, and may be kept free, if all the instruments and apparatus used are thoroughly sterilised, and if the operation is performed quickly and skilfully. For although some bacteria germs are floating in the air, the number of these is small in comparison with those that are clinging to the surfaces of surrounding objects, and very few can fall into the nutrient medium if the operator is sufficiently quick in his manipulation.

The blood serum is solidified at a temperature of from 65° to 68° C.; this is best done in an incubator, the temperature of which can be accurately regulated. The test-tube is not placed upright in the incubator, but in a slanting direction, so that the fluid serum occupies about three-quarters of the length of the tube. After about half an hour to an hour, the serum has become solidified in this slanting position, and then the tube can be taken out and placed upright. Meanwhile a drop of water has collected at the lower part of the test-tube; part of it was pressed out of the serum during its solidification, and the rest was condensed on the sides of the tube (condensation water). It serves a double purpose: it prevents the surface of the serum from becoming dry, and also keeps the air in the test-tube saturated with moisture. In order to prevent the evaporation of this water, an

air-tight india-rubber cap is drawn over those test-tubes which are not required for immediate use. Before this, however, certain precautionary measures must be taken in order to insure the safety of the contents of the tubes. As has before been mentioned, bacteria cannot pass through a stopper of cotton-wool; this is, however, not the case with the mould fungi.

It is true that the germs of these fungi, as well as those of the bacteria, are arrested by the cotton-wool, and that under ordinary circumstances they are unable to develop on account of the lack of moisture. But if an india-rubber cap is placed over the cotton-wool, in order to retain the vapour of the condensation water, the cotton-wool becomes moistened by this vapour, and thus becomes a suitable medium in which the spores of the mould fungi can develop. These fungi then grow, pushing their long thin threads through the cotton-wool, until they reach its free under-surface, when they bud off spores, which fall into the blood serum, and develop in it. This only occasionally occurs with bacteria, as they do not form such firm threads, and so are not so well able to push their way through the resistant cotton-wool. It is true that the plugs of cotton-wool were sterilised in the hot-air steriliser; but whilst the tubes are being filled, germs may fall out of the air upon them, or may be deposited upon them by the fingers of the

operator, and these germs would develop. In order to get rid of them, the cotton-wool, since they are only on the surface, may be singed in a flame or dabbed with a few drops of corrosive sublimate solution (1 : 1000). An air-tight, closely-fitting india-rubber cap, which has been lying for some hours in corrosive sublimate solution, is then immediately drawn over the cotton-wool stopper, without having been previously dried from the corrosive sublimate.

Blood serum, prepared in this way, remains unaltered for a long time, except that it gradually loses its nourishing properties; the fresher it is, the more suitable is it for cultivations; it can, however, be kept for six or eight weeks without serious loss of nutrient power. Some of the tubes, despite the greatest care, are sure to contain living bacterium and mould germs, and are, in consequence, useless. After a few days the colonies of bacteria and of mould fungi make their appearance, and then these imperfectly sterilised tubes can be separated from the others. A peculiar fatty film of cholesterin nearly always appears on blood serum; this must not be confused with colonies of bacteria.

The gelatine, prepared in the manner described above, is in the same way poured off into test-tubes, till they are about one-third full. Especial care must be taken that in filling the tube no gelatine should run down on the inside where it is touched by the

cotton-wool stopper, as in that case the cotton-wool would adhere to the tube, and thus cause difficulties. In order to prevent this, it is best to pour the gelatine first into a bottle which is shaped like an Erlenmayer's flask, only a piece of thin glass tubing, about 5 cm. long, is fused in at right angles just under its mouth. This tubing is now introduced into the test-tube, which is held in a slanting position, and the flask is tipped up until the gelatine flows into the test-tube. When this is about one-third full, the flask is again tipped up somewhat, so that the gelatine may not run out, and the tubing is drawn out, great care being taken that it should not touch the sides of the tube. However, if the hand is steady, a little practice soon enables the operator to pour the gelatine into the test-tubes from an ordinary Erlenmayer's flask.

The test-tubes may be sterilised, before they are filled, in the hot-air steriliser (see p. 39); but this is not absolutely necessary, and a great deal of time and trouble may be spared by omitting to do so, as they must all be sterilised anyhow after they have been filled. When the gelatine has been poured in, they are closed up with their cotton-wool plugs and put into a wire basket, which is placed in the steam steriliser, in which they undergo the so-called "fractional sterilisation."

The steam steriliser is a tall water-bath, which can be closed with a lid. The vessels (test-tubes, etc.)

do not come into direct contact with the water, but, when the water boils, with the steam. In order to prevent the cooling of the steam as much as possible, the sides and lid of the cylinder are covered over with felt. It is best to heat the apparatus with gas-burners,

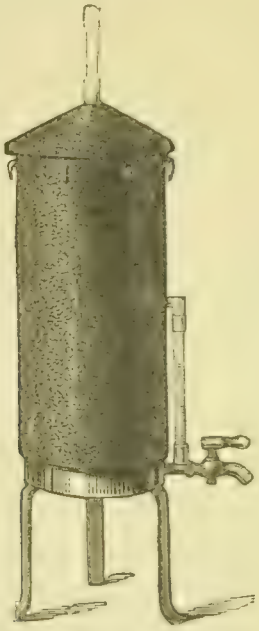

Fig. 4.—Steam Steriliser.

but paraffin lamps may be used if the steriliser is small. A convenient form is shown in Fig. 4. As soon as the water thoroughly boils (that is to say, when copious volumes of steam make their escape), the lid is placed on, after the wire basket, containing the tubes of gelatine, has been placed in the cylinder.

in which it is left for exactly five minutes. This operation is repeated for six consecutive days, after which it is certain that the test-tubes are sterilised. The gelatine may also be sterilised by boiling it for about an hour and a half on end in the test-tubes; but by this means it is deprived of its most valuable property, its power of solidification—thus becoming useless as a solid transparent medium. Boiling for five minutes, even when it takes place on several consecutive days, does not essentially affect its power of solidification, whilst it completely sterilises it. On the first day all the bacteria which are in a vegetative condition are killed in a body. Only the so-called "resting spores" are uninjured; these, on the contrary, appear to be stimulated to germination, especially as they are richly provided with nourishment. Germination takes place during the following days, chiefly during the first and second, but in a somewhat desultory manner during the later ones. But when the bacteria have once forsaken the resistant spore membrane, and have gone over into the vegetative condition, they succumb to this application of heat for five minutes; and hence the gelatine, by this method of fractional sterilisation, is completely freed from all living organisms.

If the gelatine is not required for immediate use, it is well to keep it in Florence flasks, containing about ½ to 1 litre. These must be stopped up like the test-

tubes with thick cotton-wool plugs, and must be subjected to the fractional sterilisation. The plugs are, as before, singed, and then covered over with india-rubber caps, which have been sterilised in corrosive sublimate solution. Then when the gelatine is required in the test-tubes, it is liquefied at a temperature of about 40° C., poured off in the manner described above into the tubes, after which it is again subjected to the fractional sterilisation.

If the operation has been successfully accomplished, the gelatine in the test-tubes is almost colourless, perfectly clear and transparent, and only to be liquefied at a temperature of 25° C.; if it should become liquid at a lower temperature, it has, through some accident, been kept boiling too long, and has thus lost some of its power of solidification.

The agar-agar mixture can be treated in the same way, only as it can be kept boiling from one and a half to two hours without its power of solidification being affected, its sterilisation can be completed on one day. After this has taken place the test-tubes are removed from the wire basket, and the greater number of them are placed in a slanting position in order that the agar-agar may solidify obliquely. Liquid agar-agar solidifies at a temperature of about 40°, whereas the liquefaction of solid agar-agar only takes place at 95°. Nutrient agar-agar is less transparent than nutrient gelatine when it is solid. When it is hot and liquid,

it should be perfectly clear, and of a very light yellow colour; when it is solid, it should be yellowish brown in colour, translucent, and, in thin layers, transparent.

Milk and bouillon are, in a similar manner, poured into test-tubes, plugged with cotton-wool, and then, like agar-agar, sterilised. The potato is one of the most useful of the solid media for the culture of bacteria, only it is not easy to sterilise it thoroughly. Of all the different methods which may be employed, the following appears to have proved itself the best. Firm, waxy potatoes are taken, which do not break up when boiled; they are thoroughly washed, left soaking in corrosive sublimate solution (1 : 1000) for one or two hours, and are then peeled. They are next placed again into the sublimate solution for five minutes, after which they are taken out, washed with sterilised water, and cut up into slices about 1 cm. thick, with a knife which has been thoroughly heated and is still warm. These slices are immediately put into sterilised cultivation dishes, which are then covered up with their tight-fitting glass lids, and placed in the steam steriliser. These **cultivation dishes** are flat, about 9 or 10 cm. in diameter, and $1\frac{1}{2}$ to 2 cm. deep; they are supplied with lids which fit over the sides (Fig. 5). They are at present used more frequently than anything else for the so-called plate cultivations. They are sterilised beforehand in the hot-

air steriliser at a temperature of about 160-180° C., in the same way as the test-tubes used for receiving the blood serum. The slices of potato on their dishes must be kept in the steam steriliser for at least an hour, not only to ensure their being thoroughly cooked,—bacteria, as a rule, will not grow on raw potatoes,—but also to completely rid them of all germs that might be clinging to them.

The reason why potatoes are so difficult to sterilise

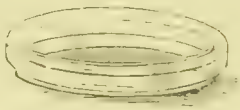

Fig. 5.—Cultivation Dish.

is this: there are some kinds of bacteria, with great powers of resistance (frequently classed together under the common name of potato bacilli), which occur in potatoes, especially so in the buds or "eyes," where they are so well protected that it is very difficult to kill them by disinfectant agents. It is therefore necessary, in peeling the potatoes, to take the greatest care to cut out all the eyes completely, and also to get rid of all black or dirty places. If there are a great many such black places in a potato, it shows that it is unsuitable for purposes of cultivation. In the spring especially it is often difficult to obtain good potatoes for this purpose.

The potatoes may be cooked without having been

previously peeled, so long as all the eyes have been carefully removed. This plan, however, is not to be recommended, as the sterilisation is not likely to be so perfect as is possible with peeled potatoes, in which every black or dirty speck can be easily seen.

These are the most important nutrient media used for bacteriological work; in a few special cases, slight modifications may be useful; these will be mentioned in the following pages.

CHAPTER III.

CULTIVATION ON PLATES AND IN ESMARCH'S TUBES.

At first glass plates were employed for cultivations, but now the cultivation dishes, of which a diagram and a description may be found in the preceding chapter, are used with great advantage in their stead. These dishes are always sterilised before they are used, by being left for two or three hours in the hot-air steriliser.

By means of these cultures on plates or on dishes, the different kinds of bacteria may be separated from one another, and cultivated apart. Thus, for instance, if we wish to isolate the different varieties which appear in meat infusion (Chap. I.), we proceed in the following manner:—A test-tube, containing gelatine, is heated to 40° C. in order to liquefy its contents, which are then poured quickly into a sterilised cultivation dish, the lid of which is held with the left hand over the test-tube, in order as much as possible to prevent the bacteria which are present in the air from falling into the tube. After the lid has been replaced on the dish, a small quantity of the meat infusion is

introduced with a sterilised instrument into the gelatine and is then well mixed with it. This mixing is most easily done by tipping the dish from side to side, care being taken that the contents should not run over the edge. When this has been successfully accomplished, a small quantity of the mixture is taken out of the dish with a sterilised wire and is then well mixed with the gelatine of another dish. Similarly, some is taken out of the second dish and mixed with the contents of a third. The instrument best adapted for carrying the meat infusion and gelatine is a piece of platinum wire, about 10 cm. long and ·8 mm. thick, one end of which has been fused on to the end of a glass rod, while the other has been twisted up into a little oval loop, about 2 mm. in its greatest diameter. This wire is specially well adapted for carrying over liquids, as a sufficiently large drop is easily retained in the loop, and in addition it can be quickly sterilised by holding it in a gas or spirit flame. In laying the wire down to cool, which takes about two minutes, especial care must be taken to prevent its touching anything, the glass rod alone being allowed to rest on the table, whilst the wire stretches over the edge. The beginner cannot be too strongly urged to take the greatest care of all sterilised apparatus and instruments, and always to bear in mind that the hands, and indeed the whole body, are covered with bacteria, and that if the platinum wire is touched or put to the

lips, in order to ascertain whether it is sufficiently cooled, it is certain to be re-impregnated with germs. Even if, in the experiment in question, it does not much matter if some species, not contained in the meat infusion, develop at the same time, still it is of the greatest importance to get into the habit of preventing the accidental introduction of foreign germs. There is nothing which requires greater neatness and care than bacteriological work, and the earlier the operator accustoms himself to the most painstaking cleanliness and care the more rarely will he have failures, and the sooner will he become a successful bacteriologist.

It is of importance that the dishes should stand as horizontally as possible after they have been filled; on this account it is best to place them on a glass plate, which has been made absolutely horizontal beforehand. This can be tested with a spirit level. According to the temperature of the room the gelatine takes a longer or shorter time to solidify; after that has occurred they can be placed in large glass dishes (see Fig. 1), which have been previously sterilised with corrosive sublimate solution.

If it is desirable to make the cultivations in agar-agar, the same method is adopted, only it is a good thing to warm the dishes beforehand to about 40° C., and above all to work very quickly, for the agar-agar solidifies at a temperature of 40°–46° C., and after

solidification has begun to take place an even distribution of the germs in the nutrient medium is no longer possible, neither does the agar-agar become evenly spread over the bottom of the dish.

The different bacteria which develop in this medium at the ordinary room temperatures grow at very different rates. It is possible that on the following day colonies of bacteria may be apparent to the naked eye; but often one has to wait two or three days, or even longer, according to the special kinds which happen to be present and to the temperature of the room. In order to convince oneself that the latter plays a most important part, it is only necessary to compare the results obtained during the hot summer weather with those obtained in spring and autumn.

With the naked eye it is difficult to distinguish the bacteria colonies from small air-bubbles; they occur as a rule in isolated positions, as the solidification of the gelatine happened to fix the germs. As they grow the different kinds may be distinguished from one another by a great number of delicate characteristics.

In gelatine cultures it is first to be noticed that whilst some sink down somewhat in the gelatine, causing it to liquefy in more or less wide circles around them, with others the liquefaction is a much slower process, and is only to be perceived after some time, and yet others do not liquefy the gelatine at all. A

number of good specific characteristics are furnished by the way in which the different species cause the gelatine to liquefy; these are however only fully recognised by degrees, even by the practised eye. Some only liquefy the gelatine just so far as the colony extends, others form only insignificant point-like colonies, which are surrounded by a ring of fluid gelatine often twenty times as great in diameter; and between these extremes there are all imaginable intermediate cases. Many turn the gelatine into a tenacious sticky substance, others into a light mobile fluid, hardly to be distinguished from bouillon. Some colonies (*Bacillus proteus*) liquefy the gelatine only on the surface and then spread themselves out in it. Of course the rapidity with which the gelatine is liquefied depends very much upon the temperature and especially upon the strength of the preparation; it is more difficult to liquefy one containing ten per cent. of gelatine than one containing only five per cent. It is therefore necessary in order to compare results obtained to work with media of the same strength.

In cultivations of agar-agar these characteristics disappear, as no bacteria have the power of liquefying it.

The different colours of the colonies also afford distinguishing traits. The colonies are only very rarely colourless and transparent; as a rule they are more or less intensely coloured; white, yellow, red, blue.

violet, or brown. The predominating colours are white and yellow; these occur in every possible tone and shade. Transparent colonies, having a distinct yellow coloration also occur, and so on. Not unfrequently the colony itself remains colourless, whilst the surrounding gelatine is coloured, generally yellow, green, or blue; or the colony may have a different colour (white or yellow) from the surrounding gelatine (green or blue).

The varying forms of the colonies afford finally a third group of specific characteristics, which are of great importance in enabling the investigator to recognise and classify the various kinds. They also show differences in position, developing either upon the surface or in the interior of the gelatine. If the bacteria do not liquefy the gelatine, they may raise button-like prominences upon its surface, or they may be nearly level with it; they may form flat, drop-like collections, or thick compact masses; they may be quite uniform, or show zone-like rings; they may be circular, oval, or quite irregular in form; sometimes their edges are clearly to be distinguished from the gelatine, sometimes they pass over gradually and imperceptibly into it; the edge may be regular, or irregular, thick or thin, tongue-like or hair-like; numerous or few runners may stretch into the gelatine. Occasionally, characteristic concentric layers, which are crossed by various radiating strands,

are to be seen. The colonies which develop in the body of the gelatine may present appearances quite different from these surface ones, even if they belong to the same species. This variation in form is due either to the favourable or unfavourable influence exerted by the air, or to the effect produced by the surrounding gelatine, which prevents the colony from spreading itself out as much as it otherwise would. Particularly interesting and important disclosures are revealed to us, if we examine the colonies with a low power ($\times 40 - 80$) of the miscroscope. For instance, in a young anthrax colony various peculiar wavy scattered masses may be observed, which might be mistaken for a bundle of curly hair; this is not seen in any other colonies. If a somewhat higher power is used, these wavy bundles are shown to consist of bacterium threads, which are lying one above the other in a mass. The edge of such a colony is generally seen with the miscroscope to have quite a characteristic appearance; it is true that sometimes it still looks sharp and clear, but generally the border, which with the naked eye or magnifying glass appeared quite sharp, now shows a notched, indented, or indistinct edge.

Such striking peculiarities are, of course, sufficient to distinguish the anthrax bacillus from all others, but most bacteria have less remarkable developments in the gelatine, and hence quite different methods

have to be employed in order to recognise and classify them. Before discussing this further it is necessary to describe another method of cultivation, which may be used instead of the cultivation on plates, and which in many cases is preferable. For ordinary use, however, the plates are the best, for they are, at any rate, more easily prepared, and, with careful handling, yield just as good results as Esmarch's tubes, whilst they afford greater facilities for observation.

Von Esmarch's method.—In this case the gelatine or agar-agar is left in the test-tubes, and, after it has been liquefied, the material containing the bacteria is quickly introduced into it. The tubes are then plugged with wadding and tipped several times from side to side, in order to thoroughly mix their contents. Especial care must be taken to prevent air-bubbles from forming, as these would greatly add to the difficulties of the later investigations. The tube is now plunged into water, which, if gelatine is used, had better be cooled with ice, and is rolled horizontally backwards and forwards on the surface, until the gelatine has solidified. Any vessel will do for the water, provided it is large enough ; but a tin dish, filled to the brim, answers the purpose best. Many prefer to place the test-tube in the dish, with its mouth stretching over the edge, whilst the remainder of the tube floats on the surface of the water. It is quite true that by this means an even distribution of the gela-

tine, and hence of the germs, is not obtained, for they naturally collect somewhat at the lower end of the tube, which is in the water, and thus the coating of gelatine becomes gradually thinner towards the mouth; but the advantage obtained by this method is that the cotton-wool cannot become wetted either by the water or by the gelatine, which can hardly be prevented if the ordinary method of rolling the tube horizontally be adopted. If, however, it is very important that the layer of gelatine should be as even as possible, it is best to proceed as follows. In order to protect the cotton-wool from the water, it can be soaked in paraffin or covered with an india-rubber cap; the test-tube is then laid on the surface of the water, and is rolled quite horizontally.

If the operation has succeeded, the gelatine should be spread in an even layer over the inner surface of the tube. If agar-agar be used, the water should not be cooled; on the contrary, it should as a rule be somewhat warmed, otherwise the medium would solidify too rapidly, and thus the layer would be uneven. Bacteria colonies develop in these tubes in the same way as in the cultivation dishes; their specific characteristics, however, are not so easy to recognise as in the latter, as observations cannot be so easily made from above.

CHAPTER IV.

CULTIVATION BY STROKE AND PUNCTURE, IN THE HANGING DROP, AND ON THE SLIDE.

After having, as described in the preceding chapter, isolated several kinds of bacteria by means of plate cultivations, it becomes necessary to keep them permanently apart, if we wish to continue their observation for some considerable time. This would not be the case if they remained for any length of time on the plates. For quite apart from the fact that very soon the gelatine would dry up, as a rule mould-fungi, or bacteria, which cause the gelatine to liquefy rapidly, make their appearance, having been either present in the gelatine from the beginning, or having gained access to it whilst the dish was opened, the result being that very soon all the colonies would run into one another. In order to avoid such a misfortune, the separate colonies must be transferred with a sterilised platinum wire into test-tubes, containing some nutrient medium, each tube, of course, being inoculated from only one colony; the so-called stroke and puncture cultivations are those most commonly used.

For the stroke cultures we use the test-tubes, in which the gelatine or agar-agar has solidified obliquely. Some of the bacterium colony is taken up with the sterilised platinum wire, and the nutrient medium is inoculated with it. The whole process of this inoculation is as follows: after having thoroughly heated the platinum wire, and having allowed it to cool for about two minutes without laying it down or letting it touch anything, the operator takes up with its point a small portion of the colony to be inoculated, seizes with the left hand the test-tube which is in readiness, containing the oblique nutrient substance, and draws out the plug of wadding with the third and fourth fingers of the right hand, laying the backs of the fingers upon it, so that the part of the plug which goes into the tube, and which it is essential to keep sterilised, should touch neither the hand nor any other object; the platinum wire is then passed very carefully into the test-tube, so as not to touch the sides with it, and is drawn gently across the centre of the nutrient medium, care being taken not to injure the surface. The platinum wire is then withdrawn, and the cotton-wool plug replaced, the stroke inoculation being completed. It is also necessary in this case to work as quickly as possible, lest germs from the air should get in. During the operation the test-tube is held horizontally, or if anything with the bottom a little higher than the mouth.

A very common mistake with beginners is to transfer too large a quantity of the colony to the medium. Only so small an amount should be allowed to remain on the platinum wire as is just perceptible to the naked eye. If too much of the old cultivation is introduced into the new, numerous cells which are scarcely living, and which are very different in appearance from the typical one, are sure to be introduced into the medium, and these may cause much confusion in the preparation, and often make the culture appear to be impure.

In these stroke cultures many peculiarities now come into prominence, which were scarcely perceptible in the plate cultivations. The colonies may confine themselves to the actual inoculating stroke, or they may spread themselves out, to a greater or less degree, until the whole surface of the nutrient medium is covered right up to the sides of the test-tube. They may flourish only on the surface, as is generally the case, or they may send down hair-like or radiating runners into the interior. In some cases peculiar skin-like ridges may be formed, and whilst some colonies shine brightly, others only do so faintly, and yet others do not shine at all; others remain as isolated drops which do not coalesce, and so on; in short, we find a series of specific characteristics, which either do not show themselves in plate cultures or are so insignificant as to escape observation.

If a puncture cultivation is to be made, a test-tube, in which the nutrient medium is horizontal, is taken, and is inoculated in precisely the same manner as the stroke cultivation, only that the platinum wire is pushed right down to the bottom of the tube. It is very important to keep the wire carefully in the middle of the medium, for if the puncture is wavy and excentric, not only does the culture have an ugly careless appearance, but very often its specific characteristics are incorrectly or incompletely shown.

In puncture cultivations the different species show greater and more striking peculiarities than in stroke cultures. To start with, some species flourish equally well upon or below the surface; that is to say, they can live with or without oxygen. Then there are a very large number of species which can only develop on the surface, or for a very few millimetres along the track of the needle; *i.e.*, they can only live where oxygen is to be obtained. The first are called facultative anaërobes, the second, aërobes; a third class, the obligatory or strongly anaërobic bacteria, do not occur on cultivation plates, and other methods, which will be described in the next chapter, must be employed for their cultivation. Moreover, bacteria which grow only on the surface also show differences in their manner of growth; some keep close to the point where the wire entered the medium, others form a small flat droplet there, and yet others form a more

or less diffuse mass at this spot. Some colonies spread themselves out in concentric layers, and others in homogeneous masses stretching outwards from the place where the needle entered; some of them are only small in diameter, whilst others spread themselves right up to the sides of the test-tube. Similarly, the ways in which they grow along the puncture line are remarkably different. Frequently a thread is formed, which may be thin and smooth, or irregular with globular protuberances upon it; sometimes it is thickest above, sometimes below; often fine hair-like processes or thick radiating branches stretch out from the more or less thick stem into the nutrient medium; sometimes also a peculiar growth is seen along the track of the wire, no thread being perceived, but only gauzy, cloud-like radiations stretching out from the central axis of the medium. Amongst some micrococci, especially those which act as exciting agents of disease, no coherent thread occurs, but only a row of globules which do not coalesce.

Further distinguishing characteristics are caused by the fact that some bacteria cause the gelatine to liquefy, and that this may take place in very different ways. Sometimes the liquefaction commences on the surface, gradually and evenly spreading downwards and towards the edge of the vessel; sometimes it occurs simultaneously along the whole length of the puncture, forming a funnel, resembling a sack or a

stocking filled with liquid. Often the bacteria fill this liquid gelatine with a uniformly thick grey mass, but sometimes the colonies sink to the lower part of the fluid, leaving the upper part quite clear. The fluid often sinks down from the surface, so that an air-bubble is drawn into the mouth of the funnel; whilst, with other species, no such bubbles are formed. Sometimes also the liquefaction first spreads itself over the surface of the gelatine until it reaches the edge of the nutrient medium, when it sinks down gradually and evenly without a funnel being formed. With some species—with those that liquefy the gelatine, as well as with those that do not—bubbles of gas may be formed, sometimes in considerable numbers, in the interior of the medium.

All these characteristics must be carefully observed in order to distinguish the different species from one another, as they afford us far more reliable data than the morphological peculiarities of the bacteria. There are a great many bacteria which, if their individual forms alone were to be considered, could not be distinguished from one another, but they show such marked and constant differences in their manners of growth in the nutrient medium, and in the form and peculiarities of their colonies, that we are bound to consider them as different species. If we have succeeded in distinguishing by means of their culture characteristics two kinds of bacteria, morphologically

very similar to each other, it is then far easier to observe slight differences in their forms, which are not marked enough in themselves to allow us to separate the two varieties from one another. On this account the culture characteristics are at present our most important guide in recognising and classifying the species.

If it is desirable to preserve a successful and characteristic stroke or puncture culture, the test-tube is melted off a little below the cotton-wool plug. By this means the culture, being shut off from the air, remains unaltered for a long time, and a collection of such test-tubes may be set aside for comparison.

It is often desirable to grow a culture upon a slide, in order to be able to observe it better or to photograph it, especially when a fairly high power is to be used. For this purpose the glass of the culture test-tube, with all its flaws and imperfections, is not at all suitable. Stroke cultivations as well as plate cultivations can be made on the slide. For the former we proceed in the following manner. The slide is sterilised, like other glass utensils, in the hot-air steriliser, and when cool is placed in a sterilised dish, like those used for ordinary plate cultivations. This dish is kept as horizontal as possible, whilst some liquid gelatine or agar-agar is taken with a sterilised pipette from a test-tube and placed drop by drop upon the slide until it is covered nearly up to its edges with an even layer

of the nutrient medium. When this has cooled, we inoculate in the usual way with the sterilised platinum wire. The development of these colonies on the slide varies somewhat from that of those in the test-tube; they are more even in their growth, whilst those on the oblique gelatine of the test-tube develop more strongly in the lower part of the tube. The greatest care must be taken to remove the lid of the cultivation dish as little and for as short time as possible, so as to avoid the entrance of germs from the air.

In order to prepare small plate cultivations on slides, the fluid to be examined, which contains the bacteria, is mixed with the liquid gelatine in the test-tube, and some of this mixture is conveyed by means of a sterilised pipette to the slide. It is of the greatest importance not to have too many germs upon the slide at once; better have only one or two than too many, for in that case the effect produced by the separate colonies is very much diminished. It is best to make three attenuations of the mixture, and to prepare several slides from each. When the slide cultivations succeed, the colonies show up much more distinctly than those on cultivation dishes. They are especially useful when a microscopic examination of the edge of the colony, etc., is to be made, for even with microscopes which have only small object-stages, each colony can be observed singly, and this is often impossible when cultivation dishes are used, especially

when the colonies are in the centre. These plate cultivations on slides must of course be preserved in sterilised cultivation dishes.

Quite the most convenient way to observe the development of bacteria with the microscope is to use the hanging drop cultivations already described. A number of slides with concave depressions are sterilised in the hot-air apparatus, together with a number of larger cover-glasses, after which they are placed with strong sterilised forceps in sterilised cultivation dishes. A cover-glass is then placed near to each depression, and the lids are put on immediately. A small drop of some fluid medium—gelatine, agar-agar, or bouillon—is taken out of the test-tube with a sterilised pipette and placed in the middle of each cover-glass. When the gelatine or agar-agar has solidified, the smallest possible quantity of the material for examination, so minute as to be imperceptible to the naked eye, is introduced into the middle of the drop, and the cover-glass is turned over and placed over the depression in the slide, so that the solidified drop hangs freely in the cavity. The edges of the cover-glass are then smeared over with vaseline, partly to keep it safely on the slide, and partly to shut out the air. This however is hardly necessary if the slide is replaced in the cultivation dish, in which case it is best only to put vaseline on one corner of the cover-glass. The opening of the pipette must be very small, so that the drops

coming from it should be sufficiently minute. There are two reasons for this. First, the hanging drop, although it must be as flat as possible, must never be so large as to touch the sides or bottom of the depression, for in that case the danger of impurities reaching the little cultivation would be much increased, and also the amount of air left in the depression would be too small; but, above all, because the development of the bacteria in deep drops could not be observed through powerful lenses which have short focal lengths. The special purposes for which hanging drop cultures are used will be explained in a later chapter.

Potato cultivations are always stroke cultivations; the material containing bacteria is taken with the sterilised platinum wire, and a stroke is made on the slice of potato (the preparation of which was described in Chapter II.), after which the dish is immediately covered up. In this case also it is a good thing to keep the cultivation dishes under sterilised bell-glasses.

CHAPTER V.

CULTIVATION OF ANAËROBIC BACTERIA.

None of the methods already described are suitable for the culture of anaërobic bacteria, *i.e.* those bacteria which thrive in the absence of oxygen. Numerous special methods have been employed, more or less successfully, but none of them are so convenient as the ordinary method of plate culture used for aërobes. Air can be excluded from the cultivation vessels by means of some substance or other, such as oil, gelatine, mica plates, etc., or it can be extracted by the air-pump, or finally it may be replaced by another, innocuous gas, such as hydrogen.

Should it be necessary to separate anaërobes from a mixture of bacteria, containing different species, varying one from the other as to their need of oxygen, it is best to employ the last method, and to make use of Esmarch's coated tubes. In order to study anaërobic bacteria a small trace of the sediment from a foul slimy ditch, or from a dung-heap, etc., is taken, and a few drops of it are put into a few centimetres of sterilised water: after these have been well mixed

together, from one to ten drops of the resultant liquid are put into one of Esmarch's tubes, prepared expressly for this purpose.

The mode of preparation is as follows: test-tubes somewhat larger than usual, and provided with well-fitting india-rubber plugs in which two holes have been bored, are filled in the usual way with gelatine, and sterilised in the ordinary manner. Through the holes in the stoppers two closely-fitting pieces of glass tubing are introduced; these are bent at right angles over the stopper, one reaching nearly to the bottom of the tube, whilst the other only just clears the stopper; the outer limbs of both are drawn out finely, so as to facilitate their being melted off later. The pieces of tubing, after their free limbs have been stopped up with cotton-wool, are placed for about three hours in the hot-air steriliser, which is kept at a temperature of 160°, whilst the india-rubber stopper is soaked for about an hour in corrosive sublimate solution (1 : 1000). When this sterilisation is completed, the fluid containing the bacteria is poured into the liquefied gelatine in the test-tube, and the cotton-wool stopper is replaced. The operator should carefully disinfect his hands with corrosive sublimate,[1] and then quickly

[1] The surest way of disinfecting the hands is to scrub them thoroughly with a brush and spirits of soap, in order to get rid of all fat, etc., and then to wash them well with corrosive

wash the corrosive sublimate from the india-rubber stopper with sterilised water, and dry it upon cotton-wool, which was sterilised with the tubing in the hot-air apparatus. The cotton-wool plug is then removed from the test-tube, and the india-rubber stopper, into which the sterilised glass tubes are quickly placed, is inserted. The longer of the two tubes is then connected with a hydrogen generating apparatus which, if the hydrogen is to be pure, must be arranged in the following manner. Chemically pure granulated zinc is placed in a small Kipp's apparatus and dilute chemically pure sulphuric acid is poured over it. Hydrogen is evolved, and is conducted through three flasks, the first containing an aqueous solution of lead nitrate, to absorb all traces of sulphuretted hydrogen; the second, nitrate of silver,[1] to absorb the arseniuretted hydrogen; and the third alkaline pyrogallic solution, to absorb the last traces of oxygen. The tube conducting the hydrogen is then connected with the one from the test-tube. The india-rubber stopper is covered over with a thick layer of paraffin to stop up all cracks and fissures between the stopper and the tubes, etc., thus making the apparatus absolutely air-tight, and preventing the hydrogen

sublimate solution (1 : 1000). The towel used for drying them must also have been sterilised in the steam steriliser.

[1] Or silver sulphate.

from escaping and becoming replaced by air, which would render the experiment useless.

The test-tubes containing gelatine are now placed in a vessel containing water at 37°–40° C., in order to prevent the solidification of the gelatine during the introduction of the hydrogen; should this however occur accidentally before they are rolled, the gelatine can be reliquefied by placing the tubes in warm water at about 40° C. The stopcock of the generating apparatus is then opened, and the hydrogen is allowed to pass through the fluid gelatine until all the air is forced out of the test-tube; this will take place in about five minutes. It is well to allow rather a longer time in the case of the first test-tube, on account of the air which is present in the tubes, etc., and which is carried along by the hydrogen. When all the air has been expelled from the gelatine and test-tube, the tubes are quickly melted off, first the one open to the air, and then the one connecting the test-tube with the generating apparatus, so that the test-tube is hermetically sealed up from the air.

The gelatine is now rolled out, either in the manner described first, *i.e.*, the mouth of the test-tube being placed over the edge of the vessel, in which case the latter must be high enough to prevent the melted off ends of the glass tubes from touching the table, or by rolling the test-tube freely in the hands under a stream of water from a tap.

If agar-agar should be used for these cultures, the experiments must be performed as quickly as possible, as this nutrient medium solidifies very quickly, and cannot be reliquefied like gelatine, for, as has already been mentioned, it only liquefies at a temperature very nearly that of boiling water, at which most bacteria are killed. The test-tube containing the agar-agar may, during the passage of the hydrogen, remain in the air, but it is better to place it in a large vessel of water at about 42° C., so that it may not cool down too quickly. The gas is then allowed to pass briskly for about three minutes, after which the tubings are melted off, and the test-tube rolled in the air, or on water of from 20° to 25° C.

It is advisable to add to the nutrient media, used in these experiments, as a rule gelatine or agar-agar, some substance which has a great affinity for oxygen. Chemically pure grape sugar is most suitable for this purpose, having the great advantage over other reducing agents which might be used (such as, for example, sodium or calcium formate), that it helps the agar-agar to adhere to the sides of the test-tube, and not to fall off even during long experiments, as alone it very frequently does. On this account, when cultivations of anaërobes are to be made, 1 % of chemically pure grape sugar is added to the gelatine or agar-agar preparations. As, however, the use of grape sugar is not injurious, but rather, as a rule,

advantageous to all bacteria, it may be always added to the gelatine or agar-agar.

This method of cultivating anaërobes is the best at present known, and indeed it leaves nothing to be desired. Even those bacteria which are affected by the smallest trace of oxygen, can be very easily cultivated in this manner. The development of the colonies proceeds very much in the same manner as that of aërobes in the dishes or in the ordinary coated tubes, though of course anaërobic bacteria generally present a somewhat different appearance.

Side by side with the colonies of strictly anaërobic bacteria, numerous other forms develop, namely, the facultative anaërobes, or the bacteria which flourish equally well with or without oxygen; for between strictly anaërobic and strictly aërobic bacteria, all imaginable intermediate grades occur. In order now to classify the different species according to their need of oxygen, and to separate strictly anaërobic from aërobic bacteria, there is an excellent method, which is called *cultivation in deep layers of solid nutrient media*. Test-tubes about 18 cm. long are filled with gelatine or agar-agar till the surface of the medium is only a few millimetres from the cotton-wool plug. The contents are then boiled, in order to expel all traces of air, and are allowed to cool until they just begin to solidify. Some of one of the colonies, grown, as described above, under hydrogen, is then

taken with a sterilised platinum wire, and the contents of the test-tube are inoculated with it, a puncture being quickly made right down to the bottom of the tube. The cotton-wool plug is then replaced, and an india-rubber cap drawn over it, or better, the upper end of the tube is immersed in fluid paraffin. All kinds of bacteria, anaërobes, aërobes and facultative anaërobes, develop equally well in these tubes, only the appearances of the cultures vary considerably according to their need of oxygen. The strictly anaërobic bacteria develop only in the lower part of the tube, whilst in the upper part, as far as the oxygen of the air can penetrate into the nutrient medium, a more or less broad band of gelatine or agar-agar remains free. The more strictly anaërobic a bacterium is, the deeper down does its development commence. Facultative anaërobes on the other hand flourish both in the upper and lower layers, or even on the surface of the medium, and we can measure their need of oxygen very well by their position in these cultures. Strictly aërobic species, which of course do not occur in the hydrogen tubes, would grow only on the surface of these cultures, there being no sign of growth along the whole length of the puncture. This method therefore affords us an excellent means of determining which of the colonies which have developed in the coated tube are really anaërobic.

This cultivation in deep layers is to be recommended,

on account of its simplicity, for the further cultivation of strictly anaërobic bacteria, and we can invariably make use of it for this purpose.

If it is wished to take specimens of bacteria from these cultures for microscopical examination, or, above all, for staining, all sorts of difficulties present themselves, amongst which the two most important are the following. Either too little of the colony adheres to the loop of platinum wire, the rest having been rubbed off the wire by the upper part of the nutrient medium as it was taken out, or so much is obtained that the difficulty of spreading the bacteria out on the cover-glass and of staining them later on is much increased, whilst the cleanliness and clearness of the preparation is much injured.

This difficulty can be overcome, in at any rate the majority of cases, in the following manner. Test-tubes which are filled for about one-third of their length, as a rule, with gelatine or agar-agar, are boiled for a few minutes in order to expel the air as much as possible, and are then laid in a slanting position in iced water in order that their contents may solidify quickly before much air can get in again. The condensation water, in the case of the agar-agar, is then allowed to run out; the medium is quickly inoculated, the material being rubbed over it as gently as possible so as not to disturb its surface, and the test-tube is inverted and placed over a tube which is bent at right

angles, and connected with the hydrogen-generating apparatus. After four or five minutes the air is expelled from the test-tube, which is then, whilst still held over the stream of hydrogen, quickly closed with a tight, well-fitting india-rubber stopper, which is without holes and which has been sterilised in the usual manner. Whilst the tube is still kept upside-down its stopped-up end is immersed in a dish of liquefied paraffin, prepared beforehand, in order to close up any fissures between the tube and its stopper, and thus to render the apparatus quite air-tight.

The success of these cultures of strictly anaërobic species depends chiefly on the exactitude, skill and quickness of the operator; they easily fail if any precaution has been neglected. With careful manipulation, however, so very little oxygen can remain in the nutrient medium, that there is no doubt but that only very few anaërobes are hindered by it in their development. If preparations to be examined with the microscope are to be prepared, it is certainly best to employ this method; but if permanent cultures of anaërobes are to be made, it is too inconvenient, and it is therefore best to make the cultures in deep layers of solid media, as described before.

In many cases, especially when it is only desired to observe the characteristic growth of a species of anaërobes in gelatine or agar-agar, another method may be adopted, which is nearly as successful as the

above, only inoculation is more difficult. The nutrient medium, which only occupies about one-third of the test-tube, is boiled in order to expel the air, and then, just as it has hardened, is inoculated. Sterilised oil is then poured in, until the test-tube is nearly full, in order to shut out the air. This method, however, is not very much to be recommended.

Species which are not strictly anaërobic may be cultivated on plates, if the gelatine is covered with a large sheet of mica just as it is solidifying. For strictly anaërobic bacteria this method does not answer, as too much oxygen gets into the gelatine before the mica is put on, owing to the large surface it exposes to the air.

If nothing but the further cultivation of anaërobes is desired, this can be effected in a considerable quantity of bouillon, to which it is best to add 1 per cent. of grape sugar. In this manner, however, the characteristic appearances seen in the solid medium cultures are lost, and it is not so easy to discover any accidental impurities, on which account cultivations in bouillon are hardly to be recommended.

Finally, anaërobes can also be cultivated in the hanging drop, if the following method is adopted: cover-glasses and slides are prepared in the manner described in the preceding chapter, inoculation is performed, and the cover-glass is laid on, a drop of concentrated caustic potash solution being placed at

the corner, and a drop of pyrogallic acid solution (1 : 5), at another; the cover-glass is then immediately surrounded with paraffin or Canada balsam. As soon as the two drops coalesce, which, if they are large enough, takes place very soon, a fluid is formed, which, having a very great affinity for oxygen, absorbs it out of the cavity in the slide, and thus the necessary conditions for the development of the anaërobes are provided.

CHAPTER VI.

CULTIVATIONS AT HIGHER TEMPERATURES.

THERE are a great many bacteria, which at the ordinary temperature of a room can either only develop slowly, or not at all, and it is therefore necessary, in order to become thoroughly acquainted with their peculiarities and characteristics, to cultivate them at a higher temperature—as a rule at blood heat. Amongst the different forms which have made their appearance so far upon our plates, probably there would be some of one kind or another, which only develop slowly at ordinary temperatures, but which flourish well at somewhat higher ones, from 20°–25° C. Above all, pathogenic bacteria, which without exception flourish better at higher temperatures, must be cultivated at blood heat.

In order to keep these cultures at as even a temperature as possible, use is made of an incubator, which is a tin box with double walls, covered outside with felt. The simplest form, which is quite sufficient for our needs, is a square box, whose top can be removed and used as a lid, and between the double walls

of which water is poured. At one corner of the box a thermometer is immersed in the water, at another a Reichert's thermo-regulator is placed, the construction of which, it may be assumed, is known, and which is able to regulate the temperature of the contents of the box very exactly. The incubator is placed upon an

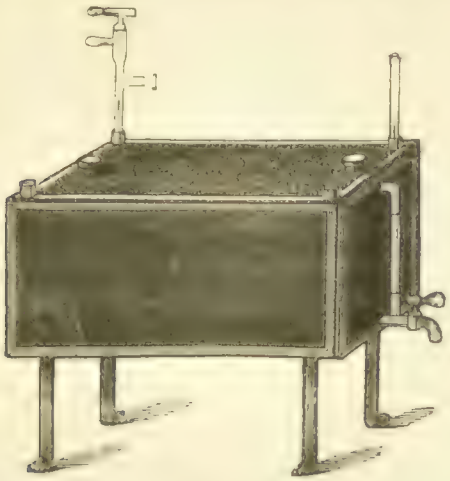

Fig. 6.

iron stand, which must be tall enough to leave a space of about 10 cm. high between the flame of the heating apparatus and bottom of the incubator. If the distance is less, not only is the heating uneven but the incubator, which, as a rule, is made of sheet zinc, suffers. Koch's safety lamps or micro-burners are the best for this purpose; but if necessary, small paraffin lamps without cylinders may be employed (Fig. 6). Before making use of the incubator, it must be care-

fully prepared. First, without regulating the supply of gas to the flame, the incubator is heated nearly up to the required temperature. The screw of the thermo-regulator is then turned, until the flame begins to become smaller. The temperature is then noted, and every two or three hours observations are taken as to whether a rise or fall in the temperature has taken place. In the first case the thermo-regulator is closed still further, in the latter it is somewhat opened, until finally the required constant temperature, in which a variation of not even $\frac{1}{2}^{c}$ C. takes place in twenty-four hours, is obtained. Then and then only can the cultivations in question be placed in the incubator.

Nutrient gelatine may be used, if a lower temperature, such as from about 20°–24° C., is sufficient. The medium in this case is prepared with 10–15% of gelatine instead of the usual 5%, in order to make it less easily liquefied. Above 25° C. no preparation of gelatine can be relied upon, as it becomes semi-fluid, so that the colonies run into one another, and lose their characteristic appearance, hence bouillon may as well, or even better, be used. In many cases it is necessary to cultivate at the temperature of the human body, 37° C. In these cases agar-agar must be used instead of gelatine.

It must however be observed, that agar-agar dries up much more rapidly at this temperature than at lower

ones, and precautions must therefore be taken to prevent this from occurring too quickly. If the cultivations are made in test-tubes, the cotton-wool plug may either be soaked thoroughly in paraffin, or may be covered with an india-rubber cap. In either case the end is gained in a very simple manner. The air in the test-tube becomes saturated with water, and after that takes no more from the nutrient medium; it is true that some vapour condenses on the sides of the tubes and then mixes with the condensation water, and that new vapour is again formed, but this is unimportant, and has no effect upon the medium. Only when the cultivations are made in media which have solidified in a slanting direction, and when they remain a long time in the incubator, does the upper portion become somewhat dry, even when it is entirely shut off from the outer air. The upper layer, which is in contact with the air, of course always dries up somewhat, and on this account when bacteria, which are very sensitive in this respect, are to be cultivated, it is a good plan to add a little glycerine (4–6%) to the agar-agar; this keeps the surface moist without interfering with the growth of the bacteria. On the contrary, it appears that a little glycerine seems to increase the nutritive powers of the medium, since many bacteria, even in puncture cultures, thrive especially well when glycerine is mixed with the gelatine or agar-agar.

As a rule, it is not necessary to make plate cultiva-

tions air-tight, as they are never set aside for so long a time as those in test-tubes. Should it, however, appear desirable to do so, especially when species that grow very slowly are to be examined, then the edge of the lower dish is carefully and quickly smeared over with vaseline before the cover is put on.

Cultivations in the hanging drop must be kept moist by covering the edges of the cover-glass with vaseline or liquid paraffin. With potato cultures it is best to place a layer of sterilised blotting paper under the slice of potato before it is cooked; this paper becomes so saturated during the cooking, that it keeps the culture moist for several days; moreover, it can always be wetted again with sterilised water if necessary. A difficulty very frequently presents itself, which is not very easily overcome. Evaporation takes place so rapidly from the large surface, that water condenses on the lid. By degrees this collects in drops, which soon become so large as to fall down on the layer of agar-agar, possibly completely spoiling the culture. This inconvenience may be got over to some extent by turning the dish over, so that the smaller surface, covered with agar-agar, forms the lid. If then the water vapour condenses below, a thing which never occurs so quickly, it will, just as it is about to turn into drops of water, be re-absorbed by the agar-agar. In many cases also it answers to sterilise pieces of blotting paper which exactly fit the lids, with the dishes, and then

after the agar-agar has been poured into the dishes to fasten them in with a few drops of the medium. This is managed as follows. The round piece of blotting paper, which should be just a little smaller than the inner surface of the cover, and which has been sterilised in the dish, is left in the cover, whilst the dish is held upside-down. The under dish is now lifted out, all the agar-agar is poured in, except a few drops, which are poured upon the blotting paper. These few drops soon solidify, and fasten the blotting paper, as a rule, firmly to the cover, which is held during the short time over the dish containing the agar-agar, in order to prevent germs from the air falling in. The whole operation requires some practice, but when rightly done, is very successful, and quite answers the purpose of rendering the condensation of the water vapour harmless. By degrees the blotting paper becomes saturated with moisture, when it clings all the more firmly to the lid. It could never happen that water would drop down from the paper to the agar-agar, for the former is capable of absorbing more water than the latter can give off. Especial care must be taken that no impurities should occur in these plate cultures, as in that case they would inevitably be spoiled, since at these higher temperatures the saprophytic bacteria grow so rapidly that they would soon take possession of the whole dish.

For the same reason the student is recommended to

close up the test-tubes with the greatest care. The cotton-wool plugs should be taken out with strongly heated forceps, held in a flame, and then rapidly re-inserted in the test-tubes. In this manner the outer impregnated portions of the plug are burnt off, and the spores, which had fallen on it since the test-tube was sterilised, are killed. It is also a good plan to heat the upper part of the test-tube strongly in a flame. In all cases the india-rubber cap must have lain for half a day in corrosive sublimate solution (1 : 1000).

In conclusion, it may be mentioned that in a great many cases a much simpler apparatus than the incubator may be used. The student can, at small cost, construct it for himself, only, of course, it would not be suitable for very accurate scientific experiments. A strong plate of zinc, of the necessary size, is placed on a strong wire tripod, and upon it a box made of strong cardboard is put, the size and get up of which may be varied according to taste. The most convenient size I found to be 30 cubic cm., outside measurement. A second cardboard box is placed in this one, smaller by 5 cm. in every direction than the first, and between the two a thick layer of cotton-wool is inserted. The boxes must lie closely upon the sheet of zinc. A hole is made, into which a thermometer fits as tightly as possible, and all chinks are then carefully stopped up with cotton-wool. A small paraffin

lamp, without a cylinder, is then placed under the zinc plate, upon which the cardboard boxes are placed, and their temperature is regulated by screwing the lamp up and down until the required temperature is permanently obtained. If the lamp burns well, and if the apparatus has been prepared carefully enough, a fairly even temperature, scarcely fluctuating for 4°, may be obtained. This is sufficiently accurate for most cultivations.

CHAPTER VII.

THE STAINING OF COVER-GLASS PREPARATIONS.

Unstained bacteria cannot easily be seen even with the best instruments, although, in consequence of their different refractive powers, they stand out faintly from the surrounding medium. The observation of these living unstained bacteria is of course the most important, but as a rule this difference in refractive power is really too small for the recognition of delicate details in their construction and form, which can be easily seen if their optical appearance is altered. This can be best brought about by staining—that is, by soaking the bacteria thoroughly in some substance which has the power of absorbing a number of colours, and of transmitting light of certain fixed wave lengths. Bacteria are specially suitable for staining, as most of them take up certain aniline dyes with great avidity, and become intensely coloured.

There are two ways of staining bacteria: either the tissue containing them is cut into thin sections, which are stained in a suitable manner (see following chapter); or the bacteria are placed on cover-glasses and

stained there. The latter method of making the so-called *dry cover-glass preparations* is as a rule much to be preferred, and is continually employed in all bacteriological work.

It is necessary to mention that the mere rubbing with a cloth is not sufficient to thoroughly cleanse the cover-glasses. The best plan is to dust them, and then to heat them thoroughly for a few minutes in concentrated sulphuric acid; after rinsing them in distilled water, they should be immersed in a mixture of equal quantities of ammonia and alcohol, and after that polished on a linen rag which is quite free from grease. These thoroughly cleansed glasses must be preserved as carefully as possible from dust; this is best done by placing them in a glass dish with a sliding cover. Before use, the cover-glass should be taken up by its outside edges with a pair of forceps, and drawn a few times slowly through the flame of a Bunsen burner or of a spirit lamp. The last traces of grease, which cling persistently to the slide, are removed by this strong heating.

We now take with a sterilised platinum wire a small portion of one of our pure cultures, care being taken to choose one that is not too old, and mix it carefully with a drop of distilled water upon a slide.

A small portion of this bacterium water is next spread out as flat and evenly as possible upon a cover-glass, and then allowed to dry in the air. It is essen-

tial, if the preparation is to be of any use, to refrain from heating it in order to dry it more quickly. If left alone, the bacteria do not practically alter their shape at all during the process of drying; but they do not adhere so firmly to the cover-glass that they cannot be easily washed off with water, and hence it is necessary to "*fix*" the layer which contains them. The simplest way to do this is to pass the cover-glass, with the side on which the bacteria are uppermost, three times through the flame of a Bunsen burner, at the same rate as the pendulum of a middle-sized regulator swings.

This fixing requires great care; if the cover-glass is not sufficiently heated, the bacteria do not subsequently stain so readily, and also are apt to become detached from its surface; if, on the other hand, it is too much heated, they almost entirely lose their power of absorbing the stain. The safest plan is to take up the cover-glass by its edges between the forefinger and thumb, and to pass it quickly through a low flame of the Bunsen burner, until it becomes unpleasantly warm.

Particles of gelatine and agar-agar must never be transferred with the bacterium material on to the cover-glass, for, although they dry well, these nutrient media do not become securely fixed, and hence are apt to become detached later, especially when warm staining solutions are added, when as a rule they carry the

bacteria off the cover-glass with them. Should it be impossible to avoid taking a minute quantity of gelatine, etc., a little white of egg should be added to, and thoroughly mixed with, the bacterium material. This is often strongly to be recommended when anaërobes, which grow in the interior of solid media, are to be examined, although, under these circumstances, the preparation never presents the same clean and pretty appearance as when none of the medium is conveyed on to the cover-glass. As concerns the quantity of material which should be used for these cover-glass preparations, it should be noticed in the first place that the farther apart the individuals are from each other, the more distinct and pretty are the preparations. On this account only a small quantity of bacterium material should be placed upon the cover-glass; it does not much matter how much liquid is added, except that, of course, the less there is the more quickly does the preparation dry. If the liquid does not spread itself out evenly over the cover-glass, but returns to the place where it was put, it shows that the cover-glass was not quite clean, but had a minute layer of grease upon it, which must be removed by heating it again in the flame and polishing it with a dry cloth. Care must be taken over all these details, if we do not wish to have a great deal of useless labour and trouble.

When the cover-glasses have been dried in the air

and fixed, they may be immediately stained, or they may be put aside, if convenient, for a time, as no change takes place in them if they are left to themselves. There are a very great many treatises on the subject of staining bacteria; however, we will content ourselves with a few good methods, which may be used in all cases, and in this chapter will only consider staining with simple aqueous solutions.

Amongst aniline dyes, only the basic ones are suitable for staining bacteria, and amongst these, fuchsine, gentian violet, methyl violet, methylene blue, and vesuvin (Bismarck brown) are the ones most commonly used. These colours are used in aqueous solutions, which may either be prepared afresh each time by dissolving the dye in water and filtering the solution, or of which permanent concentrated solutions may be made in alcohol, a few drops of which should be added to a vessel containing water. These alcoholic preparations may be kept for any length of time, whilst the aqueous ones, with the exception of that of methylene blue, soon spoil. Vesuvin, or Bismarck-brown, can only be dissolved in water, and cannot be preserved. The solution, in whichever way prepared, should be perfectly clear and transparent in thin layers—that is to say, it should not contain too much of the dye. This can be tested in the following manner. Place a watch glass over a piece of black paper, upon which a white paper cross has been laid.

If this can be clearly seen through a depth of 5 cm. of the staining solution in the middle of the watch glass, the solution is of the right strength. This, of course, only holds as a general rule; occasionally it is necessary to use a somewhat stronger or weaker solution.

Either a few drops of this staining solution is carried with a small pipette to the cover-glass, or the latter is laid face downwards upon the surface of the liquid, so that it floats. The second method frequently results in a failure at first, but with practice the student soon succeeds with it. The best plan is to take the cover-glass up by its edges with the forefinger and thumb, to bring it as close as possible to the surface of the staining solution, and then to drop it suddenly. By this method fewer stain rings are formed on the edge of the cover-glass than by the other; these rings prove themselves very troublesome later on when the cover-glass is washed. The first method is certainly the simpler; the surface of the cover-glass must be completely covered up with the liquid.

If solutions of fuchsine, or gentian violet, are used, as is generally preferable, then the preparations are as a rule sufficiently stained in from five to ten minutes; if methylene blue is employed, they take rather longer. Vesuvin is only used in certain cases, which will be mentioned later on.

If it is desirable to hasten this process of staining

the solutions may be warmed. After a drop has been placed upon the cover-glass, the latter may be carefully taken up with the forceps, and held over a low flame until steam begins to be formed. It is then withdrawn from the flame for a time, after which it is heated again once or twice in a similar manner. The liquid must on no account be allowed to boil, as, in that case, stain precipitates are formed, which can only be got rid of later on with great difficulty. If the staining solution on one part of the cover-glass should become dry, it is best to add a little more of the solution, and to apply heat for a moment, rather than to try and wash off the dried up stain with cold water. The cover-glass is then immediately rinsed in water, the best plan being to hold it under a tap until no more colour comes off. It is then laid on a slide, being firmly pressed down upon it with the left thumb nail, whilst it is dried with a soft linen cloth, after which it can be examined with the microscope. When the cover-glass is floated on the staining solution, which it is best to put in a flat porcelain dish, the latter is held over a flame until clouds of steam rise up from it; it should then be allowed to stand for about two minutes, without being heated again; the cover-glass is then taken out with the forceps and treated as before.

In successful preparations the bacteria are stained a deep red or violet, whilst the background is colourless.

The coloration is generally even, that is to say, the whole of the cell is pretty equally stained, there being no distinction between the membrane and its contents. A much less pretty result is obtained, if bacteria from older cultivations are taken. For one thing, they take up the stain much less readily, and hence are paler and less distinct, and, besides, they do not become evenly coloured ; one end of the rodlet may be deeply stained, whilst the other is only faintly so, or colourless gaps may occur in the cells, or round, deeply stained spots may be seen. These colourless gaps are either spores (*i.e.*, the resting or permanent condition of the bacteria) which, as we shall see in a later chapter, are very slightly affected by aniline dyes, or they may be actual cavities in the cell. The plasma sometimes shrinks up here and there, and cavities which do not absorb the stain are formed between its intensely stained particles. The membrane over these cavities sinks in when the bacterium is dry, and as they contain no substance capable of swelling, they do not become extended again when the preparation is soaked in watery staining solutions, so that it often appears as though the bacterium were thinner at these parts than at the coloured ones.

There are also some bacteria (such as the bacillus of fowl cholera) in which, even in the youngest cultures, a bright streak is to be seen down the middle of the cells in the stained preparations. This forms a

useful specific characteristic, by means of which those species can be distinguished from others, in which such streaks never occur, except in quite old cultivations.

It may be further observed that some species stain easily and intensely; whilst others of the same age only become coloured very slowly, and even if they are left for a long time in the staining solution, never become intensely red or violet. This is partly caused by the fact that some kinds grow old earlier than others, and hence with these much younger cultures than usual must be used; and partly that some bacteria absorb staining reagents much more readily than others. There are bacteria which only become imperfectly coloured even when they remain for days in the staining solution. These differences in their behaviour towards staining reagents are of great use in differentiating various species. Great variety is also shown in the persistency with which different kinds retain their colour when once stained.

If we make such stained cover-glass preparations of a number of species with material obtained from our pure cultures, we shall very soon notice how differently various species behave towards decolourising reagents. First, let us try the effect of a very weak decolouriser, ten per cent. alcohol. In half a minute it is seen that nearly all the species have parted with a large proportion of the staining material that they had

absorbed. Some, indeed, are nearly colourless. If they are left for a longer time in the weak alcohol, some are sure to lose all their colour, whilst others will retain it more or less, and this difference remains approximately true, even if they are left for hours in the fluid. It is true that little by little minute quantities of stain are extracted from the preparations, so that soaking for days in the weak alcohol makes them colourless. A stronger effect is produced by a more concentrated solution of alcohol, and still stronger ones by solutions of acids, as we shall see later on.

CHAPTER VIII.

THE STAINING OF BACTERIA IN HUMAN AND ANIMAL TISSUES.

In order to demonstrate bacteria in animal tissues, it is necessary to cut the thinnest possible sections of them. This cannot, however, be done without somewhat complicated preparations being made beforehand, as the tissue itself is too soft and gives way too much to the razor in cutting. The best substance for hardening the animal tissues, which are to be used for bacteriological purposes, is absolute alcohol, which we shall settle to make use of once for all in all such work.

We will suppose that the spleen of a patient who has died of typhoid has been made over to us from some hospital for examination; this is a material which is generally obtained without much difficulty. The fresher the spleen, the better; if it has begun to decompose, it is no longer any good for this purpose. We cut the spleen up into small pieces about a cubic centimetre in size, and place them into a fairly large quantity of absolute alcohol. After about two hours

we take one piece out, leaving the remainder in soak for future use. We now take a wide-mouthed bottle, with a well-fitting cork stopper, on to which we fasten the piece of material with a needle. The bottle is then filled with absolute alcohol, so that when the cork is replaced, the piece of spleen is completely immersed. The alcohol gradually abstracts the water from the tissue, and as that containing the water sinks to the bottom, fresh alcohol continually comes in contact with the material. The alcohol is changed on the next day, after which as a rule the specimen is thoroughly hardened, and suitable for being cut up into sections. Sometimes it is not necessary to put fresh alcohol, but on the other hand it is sometimes necessary to change it a second time. This difference is caused partly by the varying capacity of the alcohol for holding moisture, and partly by the varying amount of water contained in the tissue. Tissues containing much water are of course more difficult to harden than those containing little.

When the pieces of spleen are sufficiently hardened, they are taken out of the alcohol and cut up into halves, if the sections are to be made with a microtome. One of these halves, five millimetres thick, is then stuck by one of its broader sides on to a cork, which can be securely fixed into the clamp of the microtome. It is best to use a solution of gum for sticking it on, after the alcohol has evaporated. The piece is pressed

firmly on to the layer of gum on the cork, which is then placed in absolute alcohol, in order to remove the water from the gum. This generally takes place in about five hours. Fish-glue is not to be recommended, as most of the preparations sold under that name are soluble in alcohol, and thus the piece of tissue falls off. After the water has been abstracted from the gum, the tissue is quite firmly attached to the cork, which can now be screwed tightly into the clamp of the microtome. The surface of the tissue to be cut, as well as the edge of the knife, are moistened with absolute alcohol, and sections of from ·02 to ·06 mm. in thickness are made. The thinner the sections, the more easily, as a rule, are the bacteria rendered visible. A convenient form of microtome is shown in Fig. 7. A minute description of these instruments would be out of place here.

The thin sections are now taken up with a needle and a brush and put into absolute alcohol, from which they are conveyed direct into the staining solution. If typhoid bacilli are to be examined, the ordinary aqueous solutions must not be used, as they have not sufficient staining power for these organisms. A staining solution must be prepared of 100 ccm. of caustic potash solution (1 : 1000) and 30 ccm. of a concentrated alcoholic solution of methylene blue (Löffler's methylene blue). This fluid has much stronger staining properties than the ordinary aque-

ous aniline dyes, and may be kept in well closed bottles for years in a condition fit for use.

The sections, if sufficiently thin, need only remain in this Löffler's methylene blue for from five to ten

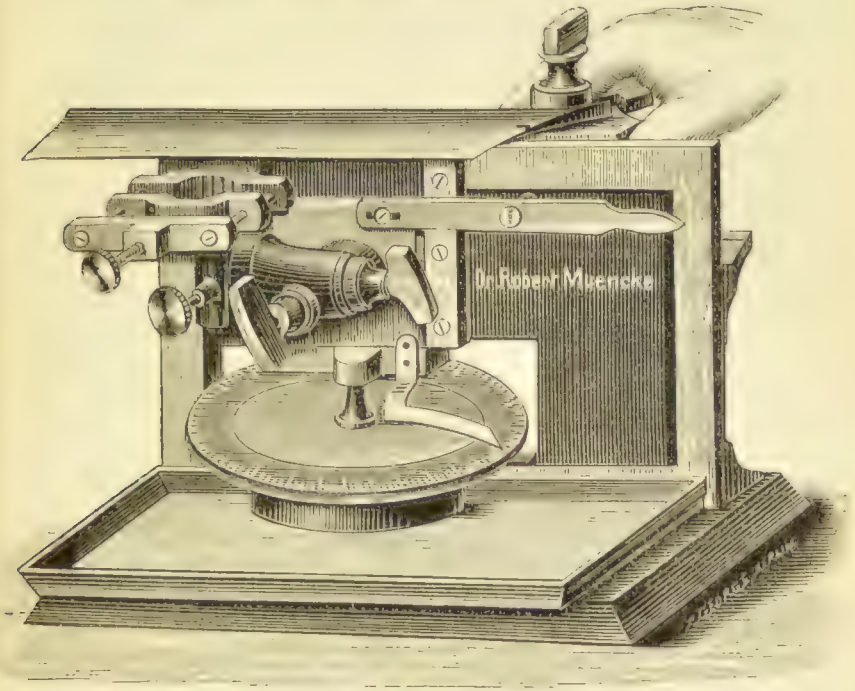

Fig. 7.

minutes; thicker sections must be left in for a longer time, even in some cases for an hour. If aqueous solutions are used, the sections must sometimes be left in for more than twenty-four hours, before they are sufficiently stained. When this has been accomplished,

the section is rinsed with distilled water, in order to wash off all superfluous colouring matter, and is then immersed for a few seconds in 1 per cent. acetic acid. After having been taken out of this weak acid, it is again well rinsed in water to remove immediately the last traces of acid. Acetic acid has the power not only of decolourising the tissue, but also the typhoid bacillus; it is therefore necessary to be very quick, if the colour of the latter is to be retained and that of the former to be only partially abstracted. The section is now transferred in water to the slide, and examined with the microscope.

If the experiment is successful, the tissue should not be entirely decolourised, but should have a faint bluish tinge; moreover the nuclei of the cells should show a distinct blue coloration. If bacteria are present in the section, they are stained a deep violet blue, that is to say, they are of the same colour as the animal tissue, only of a deeper shade. Typhoid bacilli occur generally collected together in small heaps; section after section of the spleen may be examined in vain to find them, as they occur collected together in nests in this organ. On the other hand they are evenly distributed in the intestine, of which hardly a single section can be cut which does not contain them, provided that they are present in sufficient numbers. This, however, is not always the case, and especially not in every stage of typhoid. If the operator is afraid of

decolourising the bacteria as well as the tissue with the acetic acid, the sections being thin, they may be thoroughly rinsed in distilled water. Under these circumstances the tissue is much more intensely stained, and in somewhat thicker sections the bacilli may be overlooked; but they are, however, always more intensely coloured than the tissue, and still stand out pretty well.

If it is desirable to cut up thin pieces of tissue with the microtome, it is best to fix them with gum between two pieces of elder pith, and then to place the piece so formed between two bits of cork; the whole can then be screwed into the clamp of the microtome. The method of embedding the pieces in paraffin and celloidin is not much used in bacteriological work, as it is quite sufficient if the pieces of tissue are thoroughly hardened.[1]

If a microtome cannot be procured, the student soon learns with a little practice to cut sufficiently good sections with a razor, though of course they can never be so even or so large as those cut with the microtome. It is necessary to have an excellent razor, which has been ground flat on the one side which is laid on the section and concave on the other, and which must be continually sharpened as much as pos-

[1] Minot's microtome for cutting paraffin sections is especially to be recommended.

sible on a good razor strop. It is equally important to thoroughly harden the material to be cut; only well hardened substances can be cut in thin slices by hand with a razor, and it is better to change the alcohol too often than too seldom, in order to save subsequent time and worry. The pieces of tissue may be gummed on to pieces of cork as before, in order to have something to hold them by. When a section is to be cut, the blade of the razor should be thoroughly moistened with alcohol; a rapid slanting cut should then be made. It is not a good plan to draw the razor backwards and forwards, as in that manner a jagged cut, as if made by a saw, is produced. If the razor is pressed vertically downwards, as a rule the tissue gets considerably torn. These sections are further treated just like those cut with the microtome.

Many organisms are successfully stained in sections with aqueous solutions only, such as the micrococci of suppuration, whilst others, like the tubercle bacillus, require even stronger staining solutions than Löffler's methylene blue. If it is not known whether the tissues to be examined contain bacteria or no, it is always best first to use Löffler's solution, and then if that produces no effect, the more complicated staining solutions described in the following chapter.

According to Frey, blood may be included amongst the tissues. If this is to be examined for bacteria, the same methods are employed as those used for cover-

glass preparations of pure cultures, as described in the preceding chapter. A minute quantity of blood is spread out quite evenly upon a clean cover-glass with a needle, and is allowed to dry. It is even more important in this case than with preparations of pure cultures to make the layer as thin as possible, for the drop of blood contains an immense number of blood corpuscles, which, if they are too close together, or even lying one upon the other in the dried up layer, are certain to conceal the bacteria. The cover-glass preparation is fixed in the ordinary way by passing it, after it has been completely dried in the air, three times through a flame. If the layer is somewhat thicker, it is necessary to pass it through again, as by this means the plasma and corpuscles are rendered less capable of absorbing the stain, while the bacteria are unaffected. It is a good plan to lay the fixed blood preparation for a short time in 2 per cent. acetic acid (generally about two minutes, but sometimes longer) in order to dissolve out part of the plasma and to extract the hæmoglobin from the blood corpuscles, with the result that the preparation, with the exception of the bacteria, becomes distinctly less intensely stained. After this immersion in acetic acid, the cover-glass is well rinsed with water, and then the preparation is stained in the same manner as the dry cover-glass preparations.

Methylene blue is the most suitable reagent for

these blood preparations, since it has the power of producing a greater contrast between the bacteria and the various elements of animal tissue than any other reagent. It may be used either in the aqueous or in Löffler's solution.

It is not advisable to apply heat whilst the process of staining is going on, as, although the bacteria become more quickly and intensely stained by this means, the blood plasma, and especially the blood corpuscles, are also much more coloured. If it should happen that while the bacteria are well coloured, the plasma and corpuscles are too deeply stained, the preparation may be immersed for another moment in 1 per cent. acetic acid. Many bacteria are not injured by remaining a somewhat longer time in weak acetic acid, but others can only stand it for a second. The cover-glasses, on being taken out of the acid, are immediately plunged into water, in which they are well shaken about, to remove the last trace of the acid.

Pus and the tissue-fluids, which are either squeezed out of the organs, or which during many infectious illnesses collect in various parts of the body, should be examined in a similar manner, in dry cover-glass preparations. These substances are spread out upon cover-glasses, fixed, treated with acetic acid, stained, and eventually "differentiated" again with acetic acid.

Cover-glasses on which substances rich in albuminous matter are placed, should be examined at once. If they are put to one side for only a few days even, the acetic acid has no longer the power of dissolving part of their plasma, and in consequence, the difference of colour between the tissue and the bacteria is less marked.

CHAPTER IX.

THE USE OF STRONGER STAINING SOLUTIONS.

THERE are a large number of bacteria which do not stain well, if only the simple aqueous solutions of aniline dyes are used. Either it takes an exceedingly long time to produce at all a good result, or even after several days the staining is incomplete.

Amongst the species which occur in putrid meat, etc., and of which we have made pure cultures, there are very few which do not become stained with the aqueous solutions. On the other hand, a number of fluorescent bacilli, for example, only become very badly coloured. Some of these are nearly always present in impure water, and thus material for making plate cultivations can be easily obtained from muddy ditches, etc.

Some of the colonies which develop in these cultivations are sure either to impart a greenish coloration to the gelatine in their neighbourhood, or to render it fluorescent. Generally several species of such bacteria are present, and these we test with the staining solutions mentioned in Chapter VII., and select

those which are least stained, as material for our pure cultures.

Various substances, mostly alkaline, may be added to the staining solutions, in order to render them sufficiently powerful to stain these bacteria. The most important of these substances is *Aniline*, which is a peculiar, light yellow, oily fluid. The solutions may be prepared in several ways, the simplest and most satisfactory being the following:—100 ccm. of distilled water is thoroughly well shaken up with as much aniline as it can dissolve (about 4 ccm.); it is then filtered through a good filter to remove all the unabsorbed aniline, and 5 grm. of solid fuchsine (rubine) are added to it. The greater part of the fuchsine becomes dissolved if it is allowed to remain for twenty-four hours in the mixture, which should be repeatedly shaken. The staining solution is then ready for use.

In order to stain a fixed dry cover-glass preparation with this solution, it is best to pour a few drops of it through a filter straight on to the cover-glass. If the solution is not filtered, oily stain precipitates are apt to be deposited upon the cover-glass, and as these are only removed with great difficulty, they very frequently spoil the preparation. The solution may be kept for from four to six weeks; it then commences to decompose. It is best to keep it in the dark in a bottle, which is made air-tight by means of an india-

rubber cap. Decomposition takes place much more quickly if it is left in the light. If it is desirable to still further increase its staining power, a solution of caustic soda (1 : 1000) may be added to it drop by drop, until the originally clear red fluid begins to become turbid; after this no more should be added, or precipitates will be formed. In this so-called "critical condition" the solution cannot be kept for nearly so long a time as before.

This staining solution is the most powerful which is at present known. Ehrlich's solution, which is nearly as powerful, is prepared as follows:—100 ccm. of aniline water, prepared as described above, is mixed with 11 ccm. of concentrated alcoholic solution of fuchsine. This preparation can only be kept for at most two or three weeks. Gentian violet or methyl violet may be used instead of fuchsine for either solution; methylene blue is less suitable.

Ziehl's carbolic fuchsine is a staining fluid which may be kept for any length of time, and which is very nearly as effectual as the ones prepared with aniline water. The simplest mode of preparing it is to mix 100 ccm. of water with 5 grm. of carbolic acid and 1 grm. of fuchsine. Frequently 10 ccm. of alcohol are added as well, but this is not necessary; it is true that the fuchsine is more easily dissolved by its means, but, on the other hand, the staining power of the solution is diminished. Instead of the fuchsine,

1·5 grm. of methylene blue may be used. Both these solutions may be kept for an unlimited period, and on this account are exceedingly useful, for, although not quite so powerful as the ones prepared with aniline water, yet they are quite suitable for occasional use, and are always at hand.

Now, when fluorescent bacteria are treated with one of these solutions, it will be seen that even those which only absorbed the aqueous solutions in a very imperfect manner are readily and intensely stained. Of course the others which were affected by the aqueous solutions are also stained by the stronger ones. We shall, however, be able to observe many important differences in the behaviour of the various species when we proceed to the use of aqueous alcohol for decolourising.

Most of those forms which are only stained with difficulty, retain the colouring matter more firmly than those which are more easily stained; they are able, in consequence, to resist the decolourising action of very dilute alcohol. Indeed, many kinds are not decolourised at all with alcohol.[1] To this rule there are, however, a few exceptions; the typhoid bacillus, for example, which is only stained with considerable difficulty, can be very readily decolourised.

[1] This is, of course, only relative. All bacteria are decolourised in time with alcohol, as with water, only with some species the operation may take days, or even weeks.

Upon this variation of the powers of the bacteria of absorbing and retaining stains, the method of *double or contrast staining* is based. The following experiment serves to show how this method may be used with success. A minute quantity of a colony of some fluorescent bacterium, which is not readily stained, is mixed in a drop of water upon a cover-glass with a small quantity of another colony of a bacterium which is readily stained, and which differs morphologically from the first. The preparation, after it has been dried and fixed, is then left for five minutes in aniline water solution of fuchsine. This is best done by placing a few drops of the solution upon the cover-glass, and leaving them there for five minutes. The cover-glass is then washed with water, and left in dilute alcohol until the more easily stained bacteria have lost their coloration. The cover-glass is then again washed, after which it is treated with an aqueous solution of methylene blue for about ten minutes.

When now the cover-glass is examined with the microscope, it will be seen that while the fluorescent bacteria have retained the red colour of the fuchsine, even though only faintly (the intensity varies with the species), and have not absorbed any of the methylene blue, being unaffected by aqueous solutions, the other kind have lost their red colour completely in the alcohol, and are of an intense blue colour. Thus, red

and blue bacilli are to be seen, and as the species also differ morphologically, it can be immediately recognised that the difference of colour tallies with the difference of form.

Sputum, containing tubercle bacilli, affords excellent material for this contrast-staining; it is, moreover, very easily obtained. The small yellowish cheesy nodules, which are distributed about in the thin viscous fluid of the sputum are generally exceedingly rich in tubercle bacilli. We take such a nodule, and place it upon one end of a slide; then we place another slide over the first, and rub it with a somewhat lingering pressure over the under one until the nodule is pressed out into an evenly-distributed layer. A minute quantity of this is then taken up with a platinum wire, and is spread out as evenly and thinly as possible upon a cover-glass, which is completely dried in the air, and is then fixed in the usual manner. It may be passed more frequently through the flame than most cover-glass preparations, as the tubercle bacilli are very resistant; but it is not necessary to do so. A little aniline water solution of fuchsine, or Ziehl's carbolic fuchsine is then placed on the cover-glass. If no heat is applied, the staining solution must be left in contact with the sputum for from five to ten minutes. If the liquid is warmed until steam begins to be formed, and is then kept at this temperature, only about two minutes are required.

staining. Ziehl's carbolic fuchsine also yields very good results with methylene blue, and may be recommended for examinations of sputum. In this case decolourisation is effected in the same way as when the aniline water staining solutions are used.

If a preparation of sputum be put into simple aqueous staining solutions, and then rinsed with water, the tubercle bacilli are unstained, and are therefore not to be seen, whilst the other objects, the elements of the animal tissue, and the other bacteria are coloured. Tubercle bacilli are to be distinguished from nearly all other bacteria by these characteristics, namely, that on the one hand, it is so difficult to stain them; and on the other, that it is even more difficult to decolourise them. The leprosy bacilli alone resemble[1] them in these particulars, but they are hardly ever met with in Germany, and, moreover, are not likely to be found in sputum.

It is absolutely necessary to keep strictly to one of the methods described above, if the student wishes to make himself fully acquainted with the peculiarities of the tubercle bacillus, so that he may be certain to discover and recognise it under all conditions. He is

[1] Amongst those forms which have been less frequently and fully examined are found the syphilis bacillus of Lustgarten, and the Smegma bacillus, both of which behave in a similar manner towards staining solutions; and also the bacillus of fowls' tuberculosis, which apparently may be distinguished from the true tubercle bacillus.

most strongly advised to entirely disregard all the other methods which have been recommended, although, no doubt, they are more convenient, and in the majority of cases yield good results; yet they are unreliable, and just in the critical cases may mislead him. Mention is here made of the one most commonly used, in order to point out wherein it fails, and its unfortunate consequences. Many people put the methylene blue into the acid solution, in order to decolourise and restain at the same time. This has many disadvantages. The preparation is hidden by the dye, and so it is very difficult to know just when sufficient decolourisation has taken place, and thus much depends on chance. In consequence, the decolourisation is generally either insufficient, in which case the whole preparation is stained violet instead of blue, and therefore the contrast between the tubercle bacilli and the rest of the preparation is diminished; or, as is more frequently the case, it is too complete, and even the tubercle bacilli have lost some of their colour. For even the tubercle bacilli cannot resist the prolonged action of acids, the difference shown by different bacteria in their powers of retaining stain being one of degree only. Moreover, all tubercle bacilli are not equally affected by the acid; some might be completely decolourised, and others only partially so. This would, of course, render their recognition in sputum far more difficult; and yet there is another

thing which is still more injurious. The sulphuric acid adds very much to the staining power of the methylene blue, and hence the partially, or entirely, decolourised tubercle bacilli may become coloured violet or light blue. Thus if there should be only a few present, and if these offered only a comparatively slight resistance to the decolourisation, they might possibly be, by this method, completely overlooked, as they would appear against the darker blue background only as light blue rods which might just as well belong to any other slender species of bacillus.

Similarly there are great objections to the method of allowing the layer of sputum to dry on a slide, and of fixing and staining it there. Much more heat must, of course, be employed in order to fix a slide preparation than is necessary if a thin cover-glass be used; and as in addition the slide retains the heat much longer, there is too much of a good thing. The bacilli become overheated, and in consequence lose partially, or entirely, their power of absorbing the stain, and hence it is impossible to obtain really good preparations by this means.

In the place of the 5 per cent. solution of sulphuric acid, a 3 per cent. solution of hydrochloric acid in absolute alcohol may be used. A 20 per cent. solution of nitric acid has a wonderfully quick effect; sometimes a more dilute solution answers the purpose. Nitric acid, however, must always be used with great

care, as it has a very powerful effect, even occasionally decolourising the tubercle bacilli themselves.

Gram's method of staining is exceedingly useful for differentiating similar organisms. The preparations are first treated for a few minutes with aniline water solution of gentian violet. They are next introduced into a liquid prepared in the following manner: to 300 ccm. of water one grm. of iodide of potassium and one grm. of iodine are added. (As a rule, the iodine does not become completely dissolved.) The cover-glasses remain in this liquid for exactly two minutes; after which they are rinsed with absolute alcohol, until no more stain is given off. By means of this method some species remain stained, whilst others are decolourised. It must, however, be borne in mind that many bacteria are differently affected by these reagents according to the age of the cultivation; young cultures, perhaps, yielding preparations which remain intensely stained, whilst those from very old ones may be quite decolourised. This is, for example, the case with one of the most common pus bacilli, the *Staphylococcus pyogenes aureus*.

Preparations made according to Gram's method sometimes contain minute quantities of precipitates which here and there make them indistinct. These can generally be got rid of by placing the preparation for five to ten seconds in 5 per cent. acetic acid or in hydrochloric acid solution in alcohol.

The *Staphylococcus pyogenes aureus*, for example, remains stained, when Gram's method is employed, whilst the *Micrococcus Gonorrhœæ* becomes decolourised.

These methods of staining and decolourising are especially useful for discovering bacteria in sections of animal tissues. The sections are prepared in the manner described in Chapter VIII. The best results are certainly obtained by treating the section with the aniline water staining solution rather than with Ziehl's solution, or carbolic methylene blue. It is best to use the solutions cold, because the elements of animal tissues are less effected by cold than by warm solutions, whilst the bacteria are sure to absorb sufficient colouring matter if they are left in long enough. After the staining is completed, decolourisation may be effected either by Gram's method, or by means of 20 per cent. nitric acid; the sections must then be rinsed in absolute alcohol, in order to remove all the rest of the acid and the colouring matter. If it is wished to examine them at once, it is best to place each section in a drop of water upon a slide, and to cover it with a cover-glass. If they are to be kept, they must be treated in the manner described in Chapter XII.

The method of staining the sections must depend upon the kind of organisms which we expect to find. The special treatment necessary for pathogenic

bacteria will be more fully entered upon later. If, however, we do not know at all what kinds are present, and we wish to examine the section in order to find out, the following method may be employed with advantage.

I. The sections are treated either with the aqueous or with Löffler's methylene blue solution, as described in Chapter VIII. If bacteria are present, which resist decolourisation with weak alcohol, they may be examined further by means of one of the following methods, in order to discover how they are affected by other reagents, and to effect a contrast staining. If, on the other hand, the bacteria are unable to resist the decolourising power of the dilute alcohol, they may generally still be treated by one of the following methods, or, according to Gram's method, by means of which good results are occasionally obtained, and which it is, therefore, best always to use.

If no bacteria are to be seen, it cannot yet be taken for granted that there are none present in the sections, which must be treated further in the following manner.

II. The sections are conveyed most conveniently with a platinum spatula,[1] from the alcohol into the following fluids in the order given:

[1] Metal instruments are much more convenient and handy for these purposes than the glass needles and spatulæ so frequently recommended, as they are unbreakable, and less

(1) Aniline water solution of gentian violet, or of methyl violet, or of fuchsine. The sections remain in one of these solutions for from two to ten minutes. They are then taken out with the platinum spatula, dried and put into

(2) Twenty per cent nitric acid; in this they only remain for a few seconds, if the sections are thin enough, generally until the coloration is quite changed. If the sections are very unevenly cut, no notice must be taken of the thicker parts, but before the colour is completely changed in these places, the sections must be taken out with the spatula, the superfluous acid removed with blotting paper, and placed in

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clumsy. The usual steel needles and spatulæ have the disadvantage that the metal is quickly attacked by acids, especially by nitric acid, and this not only prevents their being used for any length of time, but causes black substances to be precipitated on the section, which, as they cannot be got rid of, often render the preparation quite useless. Platinum spatulæ have none of these drawbacks, but as they are not easy to obtain in the shape which is desirable, I give here a very easy method of making them, so that the student may be able to make one himself of any shape or size that he wishes. A piece in the shape of a shovel or scoop, and of the required size is cut out of a strong piece of sheet platinum, a pointed handle of about one cm. in length being left at one end. This handle is then fused into a fairly strong glass rod. It can then be bent into a very convenient form.

(3) Sixty per cent. alcohol, in which they remain until no more colouring matter is given off from them. Each section must be carefully spread out in the alcohol with a needle, as otherwise it rolls up, or forms folds and wrinkles, which can never be wholly got rid of afterwards.

(4) The section is next examined in water. If bacteria are to be seen, although only indistinctly, on account of the too great coloration of the rest of the section, it can be treated again with acid and alcohol, as described in paragraphs 2 and 3. If, on the other hand, a contrast staining is desired, the section is taken out of the water and placed in

(5) Aqueous solution of vesuvin (that is to say, if the first staining was effected by means of either blue or violet; if, however, this was done with fuchsine, methylene blue must now be used). The sections are left in for from five to ten minutes, after which they are

(6) Washed with distilled water until no more colouring matter is given off; they are then examined as described in paragraph 4. If the sections are to be kept as permanent preparations, they must be further treated as described in Chapter XII.

This method is also especially useful for the discovery of tubercle bacilli.

III. (1) The sections are stained as in II. 1 with aniline water solution of gentian violet.

(2) They are conveyed, after the superfluous staining material has been removed, into iodine dissolved in potassic iodide solution (1 iodine, 2 potassic iodide, 300 water). After two minutes

(3) They are placed in absolute alcohol (the sections being carefully spread out), until no more colour is given off.

(4) They are examined in water.

According to Günther, the sections, after having been removed from the alcohol (III. 3), may be placed for ten seconds in alcholic solution of hydrochloric acid (3 hydrochloric, 100 alcohol), from which they are conveyed into absolute alcohol. In this way the nuclei of the animal tissues are completely decolourised, whilst the bacteria do not practically lose any of the intensity of their colour. Gram's method is also suitable for the demonstration of tubercle bacilli.

CHAPTER X.

FORMATION AND GERMINATION OF SPORES, AND METHODS OF STAINING THEM.

MANY bacteria have the power of entering into a resting or permanent condition when they form the so-called *spores*, which, when they develop inside of the vegetative bacterium cell, are called *endospores*. Sometimes, however, some or all of the members of a collection of cells or of a colony are changed directly into resting cells, and these are termed *arthrospores*.

These arthrospores occur much less frequently, and are much less conspicuous, than the endospores; moreover they do not possess the same characteristics, it being often very difficult to distinguish them from the ordinary vegetative cells. In addition, it is not often easy to get material containing them for examination, so we will leave them without further consideration, and will only consider the endospores, which for the sake of brevity we will call *spores*.

The best original material for the examination of spores is afforded us by the hay bacillus, concerning

the cultivation of which sufficient has been said in preceding chapters. But since, as was mentioned before, various different species are present in the hay infusion, which are all classed together under the common name of hay bacillus, it is better to take up with the loop of platinum wire a minute quantity of the powdery sediment, which after a time settles at the bottom of the vessel, and which consists of hay-bacillus spores, and to make plate cultivations with it. It is best to prepare three attenuations with this material, as very frequently so many spores adhere to the platinum wire, and hence so many colonies develop on the two first plates, that it is impossible to obtain material for a pure culture from either of them.

A number of slides in which depressions have been ground, together with suitable cover-glasses, are then sterilised. A drop of nutrient bouillon is placed on one of the cover-glasses, and is inoculated with a small quantity of one of the colonies from the cultivation plate. The cover-glass is then turned over, placed on the slide, and sealed up with vaseline or paraffin oil. It is now best to put the slide into an incubator, which is regulated to a temperature of about 24° C.

At first the bacilli form long threads, which, being confined by the narrow space, turn and twist about, and become broken, so that sometimes their characteristic formation is not easily seen. After a time the nutrient material is exhausted, and they commence to

form spores. This may take place on the next day, but the time varies very much according to the amount of material inoculated, to the size of the drop, and to the temperature to which they are subjected. The process may be watched for about two hours, a high power of the microscope being used with a dry lens. At first a bright spot, hardly to be distinguished from the rest of the protoplasm, which is homogeneous or finely granular, makes its appearance in some of the cells. Gradually it grows brighter and larger, until, finally, it appears as a strongly refracting body with sharp outlines about as large as the future spore. It now stands out clearly from the encircling mother-bacillus, which by degrees seems to fade away, until at last it disappears altogether. Then the spore alone remains; by this time it can be seen to be surrounded with a strong membrane.

If a few of these spores are introduced with a sterilized platinum wire into a fresh drop of sterilised bouillon which is placed in the moist chamber, after a few hours germination begins. The exact time which must be allowed cannot be stated, as the rapidity with which development takes place depends upon different circumstances, especially upon the temperature In order to watch this development conveniently, it is best to place the slides which act as moist-chambers on glass benches in the incubator, which is kept at a temperature of from 22° to 24° C. The most con-

venient benches are those made of simple glass plates, whose ends are bent back; those which are made of long thin pieces cemented together are not so good, for they are difficult to sterilise, and also are liable continually to fall to pieces.

The first thing to be noticed in the developing spore is a slight change in its refractive power; it becomes less glistening and appears to increase very slightly in circumference. Gradually the membrane, which originally was of equal thickness all over, becomes

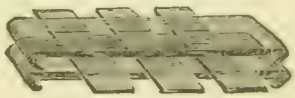

Fig. 8.

weaker in parts, being thinner at the sides, whilst at the poles it retains its original thickness. Finally it seems to disappear entirely on one side, but careful examination shows a delicate border, which is somewhat arched forwards. At last the membrane gives way at this point, and the young rodlet emerges one end foremost; it soon grows to its full size, and after from half an hour to an hour begins to divide.

Sometimes, however, the rodlet cannot get out of its spore case so easily; it cannot manage to introduce either of its ends through the aperture, and thus, as it continues to grow, has to force its way out backwards, so that at last it is curved like a horse shoe.

In the end, however, they all somehow or other get rid of their spore covering, which remains like an empty egg shell. If the conditions are favourable for growth, the rodlets, when germination is complete or with the spore membrane still covering one end, pass through a motile stage, during which, nevertheless, they divide, and gradually grow whilst becoming less active into long threads, in which again, when nourishment becomes scarce, spores are formed.

The formation of spores and their germination do not always take place in precisely the same manner; a great many variations occur, which are very useful in differentiating otherwise similar species; for example, the anthrax bacillus, which is very like the hay bacillus, breaks through its spore membrane at one of the poles and not at the side. Again, the new rodlet of the hay bacillus appears to emerge diagonally, whilst that of the anthrax bacillus comes out in a parallel direction. Really, however, this is also the case with the hay bacillus, the only difference being that the breaking down of the membrane occurs at the side and not at one pole; thus, according to their mode of germination, bacteria may be divided into two groups—those in which the spore membrane breaks down at the side, and those in which it breaks down at the pole.

A still greater number of variations are to be observed in the way in which the spores are formed in

the vegetative cell. In the anthrax bacillus the spore occurs in the middle of the rodlet, without causing it to swell out. In the butyric acid bacillus the spore again develops in the middle, but the rodlet swells considerably and becomes spindle-shaped. In the tetanus bacillus, the spore causes a considerable swelling at one end of the rodlet, thus making it appear to have a head like a drum-stick. In the kephyr ferment (*Dispora caucasica*) two spores make their appearance, one at either end, so that the rodlet assumes the form of a dumb-bell. It is, however suspected that there is a fine partition wall between the two portions of the rodlet, for it is generally considered anomalous for two spores to occur in one bacterium. In addition, there are a great number of intermediate methods, so that in almost every species some peculiarity may be observed. There are also many forms which do not form spores, or at any rate, which as yet have not been observed to do so.

Spores re-act to staining reagents like bacteria, which are very difficult to stain. If we treat a cover-glass preparation of hay bacilli which contain spores with a simple aqueous solution of fuchsine, the rodlets become stained, but the spores are unaffected, remaining as brightly shining spots in the more or less intensely red bacterium cells. On the other hand, they are readily stained with aniline water solution of fuchsine. If the solution be used hot, the preparation

should be left in for about two minutes; but the best plan is to place a few drops on the cover-glass, until it is completely covered, and then to move it continually backwards and forwards over a gas flame until steam begins to be formed. The liquid, however, must not be allowed to boil, or the preparation will not be clear.

The cover-glass is then rinsed and examined with the microscope. Now, instead of the bright spots, intensely coloured ones are to be seen, for the spores are able to absorb more stain than the vegetative cells, which have already begun to lose their vitality, and as the spores approach maturity always become less readily stained. The difference in colour is, however, not sufficiently marked; this can only be attained by means of contrast staining.

The spores being difficult to stain, are also difficult to decolourise; the preparation may therefore be rinsed with dilute acetic acid (5%) until the rodlets appear decolourised; the time this takes varies according to the intensity of the colouration. It is best to examine the cover-glass, therefore, from time to time with the microscope, so as to see when sufficient decolourisation has taken place. When the rodlets are colourless, or nearly so, the cover-glass should be immediately rinsed with water. The spores are certainly somewhat lighter, but they are still a deep red. The preparation can now be treated for a few minutes

with aqueous solution of methylene blue, which will stain the rodlets blue, whilst the spores remain red.

In this manner rodlets and spores can be exceedingly well seen. It is best to use colours that contrast well with one another. Five per cent. sulphuric or nitric acid may also be used for decolourisation, but they require much greater care on account of their powerful action.

The same difference of capacity in absorbing and retaining staining reagents is manifested by the spores as by the vegetative cells, only in a much less degree. On this account the student must find out by experiment the best method for staining the various species.

CHAPTER XI.

STAINING OF FLAGELLA.

It has already been mentioned that some bacteria manifest lively movements: it is only, however, in a few rare instances that we are able to see the organs of movement without further treatment of the preparation. Occasionally, though not frequently, they may be seen in the *Spirillum undula*, for example, which is, so to speak, a giant amongst bacteria; but even here nothing can be made out very clearly; even in the most favourable cases, we can only just see some moving points and lines at the poles of the curved cell, which indicate to us that we are dealing with the organs in question. In order to demonstrate them clearly it is necessary to have recourse to a complicated and ingenious system of staining, for they cannot be stained by any of the methods already described, as they are unaffected by aniline dyes, unless prepared beforehand in a special manner with a *mordant*.

We will again make use of the hay bacillus, in order to obtain material for our experiments, not because it

is the best, but because it is easily obtained. The following is Löffler's method of staining flagella. The surface of some agar-agar, which has been allowed to solidify obliquely in a test-tube, is rubbed gently with a minute quantity of material taken from a fairly young culture of the hay bacillus. It is most important to spread this material out as evenly as possible, and also to avoid injuring the surface of the agar-agar, for in that case small portions of the nutrient material are sure to get conveyed on to the preparations which are made later on, and which are then completely spoiled. The culture is next placed in the incubator, and is kept for about five hours at a temperature of about 35° C. Division takes place very rapidly at this temperature, about every twenty minutes; hence in about five hours the whole surface of the agar-agar is covered with a thin layer of young bacilli, which are scarcely visible to the naked eye. A minute trace of this layer is then introduced into the hanging drop, where it is examined with the microscope, in order to make sure that the movements of the bacilli have not ceased. This sometimes occurs, although the cause of their cessation is not yet fully understood.

If, then, the bacteria show continuous movements, we mix a second portion of the film very carefully and quickly with a drop of ordinary tap water upon a slide, rubbing the material as little as possible against

the slide, as the organs of movement, which are extremely delicate, may easily be torn off. Tap water is chosen because the bacteria are so extraordinarily sensitive that they often cast off their flagella if placed in distilled water. We now place small drops of water upon a number of cover-glasses, which have been sterilised and heated as before described (p. 91), and with the platinum wire introduce into each of them a minute quantity of the water on the slide which contains the bacilli. The following precautions must be most carefully taken if the experiments are to succeed :—

1. The cover-glasses must be absolutely clean, in which case the drop of water will spread itself out evenly over the surface, and not run back or refuse to adhere to any portions of it. The smallest particles of dirt become stained during the following experiments, and then may completely conceal the faintly stained flagella.

2. No trace of agar-agar should be transferred to the preparation, or the result is the same as if the cover glass were dirty.

3. There must not be too many bacilli upon each cover-glass, for they should be scattered, and the distance between them should be greater than the diameter of the bacilli themselves.

The drop is now spread out upon the cover-glass, and is allowed to dry in the air. The greatest care is

necessary in fixing these preparations. If they are passed three times through a flame, the rate being determined by the pendulum of a regulator, the preparations as a rule are a success. It must however be borne in mind that the flagella are of much more delicate construction than the cells, and if the above method does not succeed, the cover-glass may be taken between the forefinger and thumb, and passed quickly through the flame, until it just begins to be unpleasantly warm. If they are too much heated, flagella invariably lose all their power of absorbing stain.

After having been fixed, the preparations must be treated with the mordant, which is prepared in the following manner: 5 ccm. of a cold saturated solution of ferrous sulphate (green vitriol, sulphate of iron, $FeSO_4$) are poured during constant stirring into 10 ccm. of tannic acid solution (20 grm. tannic acid, 80 grm. water). To this mixture 1 ccm. of a concentrated aqueous solution of fuchsine is added; an alcoholic solution may be used, but it is not so good. The mordant, however, can only be used in this form for a very few species. In some cases it must be acidified, in others rendered alkaline, and often this must be done very exactly. Each bacterium requires for the staining of its flagella a specially prepared mordant, the acidity or alkalinity of which can only vary to a very limited degree. We prepare by titration

with standard oxalic acid solution an exact 1 per cent. solution of caustic soda, and also a solution of sulphuric acid in water of such a strength, that one ccm. of the latter exactly neutralizes one ccm. of the former. In order to preserve the caustic soda solution for a considerable time, it is best to fill several small bottles up to the brim with it, to close them with well-fitting india-rubber stoppers, and to fasten them down with paraffin. In this manner the solution may be kept in perfectly good condition for a considerable time.

In order to stain the flagella of the bacillus subtilis, twenty-eight to thirty drops of the 1 per cent. sodium solution must be added to 10 ccm. of the mordant. After these have been thoroughly mixed, a few drops are placed upon the cover-glass, until it is covered right to the edge with a single arched drop. If the cover-glass is not completely covered, ridges of dried mordant are formed, which—as very frequently they cannot be removed—are liable to spoil the preparation. The cover-glass is then held high up over a lowered Bunsen-burner flame, and is moved backwards and forwards until the liquid just begins to steam. On no account should it be allowed to boil, for if bubbles once form, the preparation as a rule is spoiled, a fine precipitate of mordant being formed, which becomes stained later on, and forms a rose-coloured background, from which the bacilli can hardly be distinguished. The cover-glass should only be warmed

for about a minute, after which it should be thoroughly rinsed under a tap, or with a wash bottle, in order to completely free it from the remains of the mordant. Small particles of the mordant are apt to cling very closely, especially on the edges of the cover-glass; if this should occur, it is best to wash the cover-glass in absolute alcohol until they completely disappear. The alcohol soon evaporates in the air, and then the cover-glass ought to appear clear, except for the portion which is covered by the layer of material containing the bacteria; this should be grey or reddish grey.

The most suitable solution for staining is the aniline-water solution of fuchsine, prepared as described on p. 111; a few drops are passed through a filter directly on to the cover-glass until it is completely covered, as it was with the mordant. It is then similarly warmed for about a minute, and afterwards thoroughly washed for a long time with a stream of water, until no more colouring matter disengages itself. This may sometimes take a long time, but is absolutely necessary if the preparations are to be faultless. The cover-glass can now be examined with the microscope.

If the preparation is successful, the bacteria are of a very dark red, almost black colour, and also appear much thicker than when stained in the usual manner: this is because by this method their gelatinous sheaths, which previously remained unstained, are

also coloured. A varying number of fine undulating curved threads are also seen stretching out in an irregular manner from the bodies of the bacilli; these are much less intensely stained, and in the most successful cases only appear a dark pink. Sometimes they appear to be attached to only one point of the bacillus, sometimes they are distributed over the whole body, sometimes they are very long, sometimes short, sometimes more on one side, sometimes all on one pole; in short, there may be great variations in the way the flagella grow even in one species. On the other hand, however, there are usually quite distinct features which are constant to the various species, and which are sometimes of the greatest use in distinguishing them one from another. We find, if we examine especially for it, that in a given species the greater number of the flagella are distributed over the whole body, or that they are in a bunch at one pole or at both poles, or that only one or two (rarely three) occur at each pole.

The following bacilli amongst others have numerous flagella distributed over the whole body: The typhoid bacillus, the bacillus of blue milk (*bacillus cyanogenus*), the potato bacillus (*bacillus mesentericus vulgatus*), the bacillus of malignant œdema, the bacillus of Rauschbrand, the *bacillus subtilis*, the ferret plague bacillus, the *bacillus proteus*, etc.

The following have only one or two flagella at the

poles: The *bacillus pyocyaneus*, the *spirillum Metschnikoffii*, the cheese spirillum (*spirillum tyrogenum*), the *spirillum choleræ asiaticæ*, the *spirillum Finkleri*, etc.

The following have numerous polar flagella: The *spirillum undula*, *spirillum rubrum*, *spirillum concentricum*, and also numerous other species of spirilla found in marshy or stagnant water. The *micrococcus agilis* has also several flagella, which possibly originate at one point.

The flagella of the blue milk bacillus are the most easily demonstrated; the mordant in this case may be used alone without the addition of either acid or alkaline solution; but the flagella are also stained, if on the one hand twenty drops of acid, or on the other fifteen drops of caustic soda, are added to the mordant. The staining is most successful if from eight to ten drops of acid are added. With this bacillus it is not so important to prepare the mordant quite accurately; with many other species, however, this is not the case. For example, in dealing with the typhoid bacillus, great exactitude is required, exactly 22 drops or 1 ccm. of the sodium solution must be added to the mordant, one drop more or less is sufficient to prevent the flagella from becoming stained. In order to determine the exact quantity of acid or alkali which it is necessary to add for different species, experiments must be made in each case; according to Löffler the

amount required to render the flagella visible is, to every 16 ccm. of mordant add for:—

Spirillum choleræ asiaticæ . .	1	drop of	acid.	
Spirillum rubrum	9	drops	„	
Spirillum Metschnikoffii . . .	4	„	„	
Bacillus pyocyaneus.	5	„	„	
Spirillum concentricum . . .	0	„	„	
Bacillus mesentericus vulgatus .	4	„	soda	solution.
Micrococcus agilis	20	„	„	„
Typhoid bacillus	22	„	„	„
Bacillus subtilis	28–30	„	„	„
Bacillus œdematis maligni . .	36	„	„	„
Rauschbrand bacillus	35	„	„	„

The mordant can be kept for a longer time if neither acid nor alkali be added to it. The addition of the soda especially causes the formation in a few days of copious precipitates. In spite of this, the mordant may still be used, but quite clear preparations are no longer to be obtained with it.

CHAPTER XII.

MOUNTING OF PERMANENT PREPARATIONS.

If a preparation has been successful, it is often desirable to preserve it. It must, however, be clearly understood from the outset that permanent preparations are only, so to speak, mummies, and that a study of them cannot replace the observation of living bacteria. Even stained preparations examined in water look very different from permanent preparations mounted in Canada balsam. The cause of this is that all the water has been abstracted from the latter, and hence the bacteria have shrunk up. If we compare such a preparation with one lying in water, both of which were made with material from the same cultivation, the difference in the size of the bacteria is easily seen. On the other hand, as a rule, their forms remain unchanged; it is only when the preparation is imperfect that distortions occur. Moreover stained preparations can only be kept a limited time; the colours gradually fade, and after a few years become so faint that although the bacteria themselves are still to be seen, it is impossible to

distinguish clearly any minute points in their structure.

Nevertheless a good collection of bacteria preparations is of great use. A large number of species may be quickly glanced over, in order, for example, to compare them, and species may be examined, of which perhaps no cultures are just then to be obtained. Very often too, interesting stages of development or peculiarities of growth or differences in position are preserved, such as probably will not readily be obtained again ; and finally, by means of a good collection of such preparations, the student is able to recapitulate quickly the important points in the life history, the development, and other peculiarities of a certain species.

The collection, however, is really only of great use if it is systematically arranged. Above all things, the student should beware of saving imperfect preparations and of trying by their great number to atone for their imperfections. None but absolutely perfect preparations are worth preserving. Further it is best at first to aim at getting several specimens of a few kinds in different conditions and stages of development, rather than to collect as many different kinds as possible. For instance, the hay bacillus affords a large amount of suitable material. There is great variety in the vegetative condition alone, although within narrow limits ; these bacilli occur in the form of long threads, of single cells, either motile or motion-

less, and of short rodlets, consisting of only a few joints. The motile cells may be stained with simple staining solutions, or, if it is wished to render the flagella visible, by Löffler's method. Further, during the processes of germination and of the formation of spores, they present a series of conditions which are well worth the trouble of preserving. Also it may be observed that the different nutrient media exert an influence over their form, especially over the thickness of the rods.

If, after examining a dry cover-glass preparation in water with the microscope, it seems worth preserving, it must be carefully lifted, not drawn, off the slide. If it does not come off easily, a drop of water should be placed at its edge; this penetrates between the cover-glass and the slide, and makes it easy to separate them. If the cover-glass is drawn off, the preparation is usually spoilt. The cover-glass is then put down with the side on which the bacteria are, uppermost, and is allowed to dry in the air; after that it is passed through a flame, to drive off the last traces of moisture, and is then laid in a drop of Canada balsam on the slide. The Canada balsam is best procured in the form of solid pieces as hard as glass; it can be dissolved in xylol; other substances in which it dissolves are less suitable, as they decolourise the preparations. It does not much matter if the preparations are made on round or square cover-glasses;

the former, however, are to be preferred, as they are more easily sealed up, and, besides, they look nicer.

Canada balsam takes a long time to dry; in winter and in damp weather it may take months before the outer layer of balsam becomes quite hard like glass. Only then can it be covered over with a layer of varnish which serves both to protect the balsam from the air, and also from the oil, in which the preparation later on may be immersed, and which would otherwise dissolve away the balsam, and thus the preparation would be endangered. The most suitable varnish for this purpose is the so-called spirit varnish.

If after having examined some sections in water we decide to preserve them, we place them in absolute alcohol, being careful to spread them out immediately on the spatula, to prevent them from rolling up and becoming distorted. After having been placed in fresh absolute alcohol, they are put into xylol, in order to remove the alcohol; the superfluous xylol is then dried off with blotting paper, and they are placed in a drop of Canada balsam. Especial care must be taken to observe that all the water was absorbed by the alcohol, and that this in its turn was completely removed by the xylol, otherwise the preparation will become cloudy when it is placed in the Canada balsam, and will be thus quite spoiled. If the sections cannot stand immersion in alcohol without becoming decolourised, it is best to press them

firmly down upon the slide and to leave them there to dry. When the section is absolutely dry, a small drop of xylol is placed upon it, in order to preserve it from the air; after it is quite saturated with the xylol, it is wiped; a sufficiently large drop of Canada balsam is placed upon it, and the cover-glass is put on. Here again the spirit varnish is used after the balsam is quite dry. The best plan, however, is to dehydrate the sections by repeated immersions in aniline oil. The colours are unaffected by this reagent, and yet the section is soon completely freed from water. The aniline oil must be frequently changed, and then when the sections are quite dry, they are treated as before with xylol, before they are imbedded in Canada balsam.

The preparations must be accurately labelled. English slides are the best for this purpose, as they afford space for a label at each end. At the one end the name of the organism, the origin (section through a lung, pure culture, etc.), and the date may be entered; and at the other, the method of preparation used. Special cabinets for containing slides may be obtained.

CHAPTER XIII.

BACTERIOLOGICAL EXAMINATION OF WATER.

Even not counting the exceptional cases, when the presence of bacteria in water is suspected during an epidemic, the bacteriological examination of water is of great practical importance. Chemical analysis alone is not sufficient; for, as regards one of the most important points, *i.e.* the determination of the amount of organic matter present in the water, it is not to be relied upon. If the quantity held in solution be small, neither its composition nor its exact amount can be gauged by the ordinary methods with any degree of accuracy.

Bacteria form a delicate test for this organic matter, for just those substances which originate in human habitations, the products of decomposition of organic substances, which get into the water from dung-pits, drains, dirty canal water, water from manufactories, etc., afford them excellent nourishment, whilst other organic substances, such as those from peat heaps or bogs, are useless to them. It is true that very probably the peaty or boggy water contains far more

organic material than that which has been contaminated by organic matter from drains, etc., yet in spite of this, the bacteria multiply much more rapidly in the latter, which is richer in nourishment than in the former, and in addition a large number of kinds develop in the drain water, which cannot live in the peat water.

Thus, at the outset, we are in a position, in the greater number of cases, to determine, according to the number of kinds present in the water, whether its organic impurities are the products of human habitations, or whether they proceed from other less dangerous sources, and it is now to be explained how this knowledge may be utilised in the hygienic analysis of drinking water.

Very frequently the aim of the examination is to determine if a certain definite pathogenic organism, generally the typhoid bacillus, is present in the water or not. Now, although it cannot by any means be stated that it is impossible to demonstrate the typhoid bacillus in water, for indeed it has been accomplished in a few cases, yet it is only possible under especially favourable circumstances, for a thousand samples of water, which is suspected of containing typhoid germs, may be examined with hardly a single successful result. The explanation of this is simple. When typhoid bacilli, by some means or other, first get into the water, they are not evenly distributed throughout

it, and thus, especially if there are only a few of them present, it may easily happen that there are none in the few cubic centimetres of water used for the plate cultivations, whilst at the same time there may be sufficient in other portions to cause infection. The most important fact however is this: that between the time of infection and the outbreak of the disease a considerable period of time frequently elapses, so that it is possible that although a great many bacteria were present in the water at the time of infection they may have disappeared a long time before the examination of the water takes place. This is easily to be understood when the water passes along pipes, for it soon flows away, and perhaps only an hour after the entrance of the bacteria they may have been all carried away. In wells the case is somewhat different. If the bacteria do not find suitable conditions, they sink to the bottom and die. If therefore the sample is taken from the upper water of the well, it may be quite free from bacteria, whilst there may be living specimens still present in the lower portions of the water.

We must therefore once for all give up in such examinations the hope of demonstrating the typhoid bacillus; and must regard its discovery as a special piece of good fortune. Nevertheless in these cases the bacteriological examination of the water affords us certain indications on which we must base the

opinion formed of its hygienic properties; that is to say, we may discover by these means if the water is capable of harbouring typhoid bacilli.

It is next necessary to make clear to ourselves how the typhoid bacillus spreads. Although it is not one of those bacteria which can develop only in the animal body, but is capable under certain circumstances of developing even in our climate outside of the body, yet this very rarely occurs, especially as the bacilli are exceedingly liable to be overwhelmed and completely covered over with a luxuriant growth of saprophytic bacteria. On this account only sporadic cases of typhoid can be ascribed to these "free-living" bacteria, if we may so use the term. Should however one such first case of typhoid occur in a neighbourhood, there is a possibility that a greater or smaller epidemic may ensue in consequence. Typhoid bacilli pass out exclusively with the excrements of infected persons, and if these are not disinfected, but are cast, containing, as they frequently do, great quantities of the bacilli, into cesspools or down drains, the fetters with which the pestilence might have been bound have escaped from the hand of man. Several different contingencies may occur; the typhoid bacilli may be carried away in different directions, generally impossible to trace. If the opportunity is afforded us of examining carefully a number of farm-yards in the country, we find that in a great number of cases it

appears probable that the typhoid bacilli get into the springs directly from the cesspools. Very often too the springs are in a condition which at the least cannot be called clean, and are in addition frequently situated very near to the privy. Small crevices in the soil, holes caused by mice, rats, or even by earthworms, are sufficient to establish a communication, or we may find that the cesspools are too shallow, or so badly constructed, that they overflow during heavy rains, so that some of the fluid enters into the spring, an accident which unfortunately occurs only too frequently. Under any of these conditions, of course, if typhoid bacilli are present in the cesspool, they also enter the spring, and thus the one case of the disease can give rise to a number of others.

It is clear from former statements that the presence of the typhoid bacillus in the water cannot be detected in the majority of cases. But the bacteriological examination of the water enables us to ascertain with some degree of certainty whether such a communication as described above has taken place, and hence, whether it is possible for typhoid bacilli to get into the water or not. If there is such a communication, a great number of other kinds of bacteria are to be found in the water, which have come from the cesspools or privies, and which can be easily recognised by their power of decomposing albumen and of energetically liquefying the gelatine of the culture-

plates. These organisms not only are able to exist much longer and better in water than the typhoid bacilli can, but they also enter in much greater numbers, and, in addition, they make their way into the water on every possible occasion, whereas the typhoid bacillus is only comparatively rarely present in the cesspools, and so can only comparatively rarely get into the spring. Thus these bacteria, which may be designated as putrefaction bacteria, may almost always be demonstrated in such water in great numbers. Water which contains great quantities of these putrefaction bacteria, must in consequence, under all circumstances, be suspected during an epidemic of having functioned as the vehicle for typhoid bacilli, or at any rate must be condemned as being capable of so functioning, should an epidemic break out. All this cannot be ascertained by chemical analysis, and hence our judgment of the water must be chiefly based in such cases upon the results of the bacteriological examination.

After this dissertation upon the aims and usefulness of the bacteriological examination of water, we will turn our attention to its practical execution. It may as well be mentioned here that a great many different methods may be employed; we will however devote ourselves to one in especial which is easily carried out.

The selection of the sample of water for examina-

tion is to be done with great care. Unless very great exactitude is required, glass flasks containing about 100 grms. and provided with ground glass stoppers, may be selected for the reception of the water. The stoppers must fit so well, that it is impossible for water to trickle out of the flasks, even if they are kept upside down for hours when full. If it is necessary to sterilise these vessels quickly, it may be done by rinsing them and their stoppers with corrosive sublimate solution, and then keeping them closed with a sterilised india-rubber cap drawn closely over the stopper. If there is no such hurry, the closed flasks may be kept in the hot-air steriliser for some hours at a temperature of about 160°. In this case, the stoppers must not be too firmly inserted in the flasks, as otherwise these may crack. It is very important to make sure, before making use of them, that the flasks which have been sterilised in this manner are quite sound. After they have cooled, a sterilised india-rubber cap should be drawn over them as before.

However, although this method is so simple, and quite answers the purpose in most cases, there are some drawbacks to it, which may make an accurate examination impossible. Although the stopper may appear to fit well, yet there may be a small chink or crevice between it and the flask, sufficient to establish communication between the water and the outside

air. Thus germs of species, which before were not present in the water, may make their way into it.[1]

With care, of course, this can only rarely occur, still, when accurate analyses are required, a method must be employed, which excludes the possibility of such accidents.

Suitable vessels, which satisfy all requirements, may be very easily prepared. Thin test-tubes are selected and are heated strongly at about their middles in a gas flame or better in a blow-pipe flame. The heated part is then drawn out into a thin tube of about 5 to 10 cm. long. The test-tubes are then stopped up with plugs of wadding, and are sterilised in the hot-air steriliser. By means of this simple arrangement we may dispense with all the various kinds of vessels which are recommended for the reception of the samples of water.

If the sample is to be taken from a conduit pipe, the water must be allowed to run for a few minutes; if a spring water is to be examined, it must be pumped up for at least five minutes, to get rid of all the germs, which are sure to be present in the pipes. If the sample is to be taken from a draw well or from standing water, it must not be derived from the sur-

[1] That the flasks if properly rinsed are uninjured by the sublimate, has been proved by the author by means of 2,000 experiments. The best methods may fail in inexperienced hands.

face. The sterilised vessels with their stoppers should be thoroughly freed from corrosive sublimate, by rinsing them carefully four or five times with water from the same source as that to be examined. On no account must other water be used lest germs not present in the water to be examined should be introduced into the vessels, and thus all the good of the sterilisation be annulled. Only when every trace of the sublimate has been removed can the test-tube be filled with the water; it should then be immediately closed up with the stopper, covered with the india-rubber cap, and labelled.

If a flask is used, which has been sterilised in the hot-air steriliser, it is simply filled and closed, but only half of the lower part of the prepared test-tube should be filled, and the wadding stopper replaced, until it is convenient for the upper part to be melted off. This must of course take place as soon as possible; if the weather prevents it being done on the spot, the operator must go as quickly as possible to the first convenient place, and heat the thinly drawn out portion of the tube in the flame of a spirit lamp, until both parts can be melted off, without being much drawn out. Two precautions are necessary in the operation, the tube must be very gently heated at first, in order to expel all the moisture from the thin part, and further it must on no account be shaken, or roughly moved until it has thoroughly cooled lest

water should reach the warm portion. The tube would be sure to crack if either of these precautions were neglected, and even if the crack were too small to be seen at once, it would be sure to cause the destruction of the tube during transportation.

If the interval between the taking of the samples and their examination is to be short, the test-tubes may be taken away simply packed in a small box. If, however, the examination is to be very exhaustive, and an interval of several hours or even days must elapse before it can take place, the sample must be protected in a special manner against the influence of time and of the altered outward conditions in which the bacteria find themselves. It must be taken as a general rule that the bacteria, if left to themselves, multiply rapidly in the sample, so that after a lapse of several hours there are sure to be many more germs in proportion in the sample than there were originally in the water of the well, and on that account the judgment formed of the water, if it rested principally on the number of germs the sample contains, would be quite incorrect. There is, however, a way to prevent this multiplication of the bacteria, that is, by keeping the tubes sufficiently cool. If the temperature sinks below a certain point, although the vitality of the bacteria remains unaffected, no division occurs with most species, whilst with the remainder it only takes place very slowly. This temperature varies with different species, but we

can for our purposes consider that it is between 6° and 8° C. It is therefore best to pack the samples in ice. There are a great number of different kinds of apparatus which have been recommended for this purpose; they certainly answer the purpose for which they are intended, but they are not always easily obtained. On the other hand, a suitable apparatus, which is equally convenient, may be easily made everywhere. Small tins, such as those used for packing cocoa, containing about from 100 to 250 grms., are selected, and in these the test-tubes are packed with wadding, so that they cannot be broken during transportation. The lids are put on, and each tin is placed in a larger one of 500 to 1,000 grms. capacity, according to the size of the smaller one. The larger tin should be prepared beforehand for the reception of the smaller, by having strands of strong wire bent into meshes and fastened securely over its bottom and sides, so as to make a safe support for the smaller one, and to keep it quite securely in its central position in spite of even violent shaking. The large tin is then filled with small pieces of ice, as much water as is absolutely necessary to fill it up is added; some more bent wire is then placed over the small tin, after which the lid of the larger one is put on, and is carefully gummed down, so as to prevent the water from running out.

Even in summer the temperature inside the small tin is sure not to rise over 6° C. in twenty-four hours.

The samples may be now sent by post, packed up in boxes. On no account should more than five test-tubes be packed in the smaller tin. The first time this operation is attempted it is probable that in one respect or other some mistakes will be made, but then the student will learn to avoid them another time.

Plate cultivations are now made from these specimens, preferably in the double dishes described before. These are sterilised in the hot-air steriliser, as well as a sufficient number of pipettes, each of which has one cubic centimetre graduated in tenths. Nutrient gelatine (10% gelatine) is generally the most suitable nutrient medium, agar-agar is only to be recommended when a high temperature is necessary.

The fluid gelatine is poured into the double dishes, which are immediately closed (the cover must not be laid down meanwhile), and a small quantity of the water sample is taken up with a sterilised pipette. If there is no means of judging beforehand whether the water contain many or few bacteria, it is best to prepare three dishes. In the first of these 1 ccm. of water is added to the gelatine, in the second $\frac{1}{2}$ ccm., and in the third $\frac{1}{10}$ ccm. The covers should always be replaced as rapidly as possible and then the gelatine and water are thoroughly mixed by moving the dish carefully from side to side. They are then laid on as horizontal a surface as possible, until they become cool, and the gelatine has solidified; they may then

be placed one on the top of the other. In order to make quite sure that no contamination from the air can take place, the dishes may be placed upon a sterilised glass plate under a sterilised glass bell jar. As a rule, however, this precaution is superfluous, for the impurities arising from contact with the air are generally of a very harmless nature, and usually do not affect the opinion formed of the water.

In the course of the next few days, the length of time varying according to the temperature and the species present, the colonies make their appearance. They soon become sufficiently large to be seen with the naked eye. Since, however, different kinds grow at very different rates, it can only be assumed about eight days after the appearance of the first colony that all the bacteria have developed, to which, under these circumstances, development is possible. Of course it may happen that all the colonies develop at about the same time, if they all belong to the same species, or to ones that grow at about the same rate. This, however, may be considered as an exceptional case, and it is wise to keep to the given time of eight days before examining the plates to find out the number and kinds of the colonies present. It may, of course, also occur that even in the plate which was inoculated with only $\frac{1}{10}$ ccm. of water, so many colonies develop that an examination is absolutely necessary earlier, for otherwise the colonies, which originally were separate,

would run into one another, and thus render a correct statement of their number and varieties impossible. There is no need of hesitation in the matter, for if so many bacteria are present as to render an earlier examination necessary, it may be taken for granted that the species which would develop later on are, comparatively speaking, of only small importance.

The real examination is founded upon two considerations. First, note must be taken of the number of the colonies which have developed; and secondly, the number and character of the species present must be most accurately observed.

If there are not many colonies, they may easily be counted; if, however, there are a great many, simple counting could hardly be relied upon, and in any case would take up a great deal of time. If there are several thousands of colonies, one more or less does not make much difference, and they are reckoned up only in round numbers. There is a simple apparatus which very much simplifies this matter of counting the colonies. This is illustrated in Fig. 9. It consists of a wooden stand upon which a slate is fixed; over this rests a glass plate, which is divided into square centimetres, and which is supported on little blocks. Most of the glass dishes are about 60 square centimetres in area, that is to say about 6 centimetres in diameter. The culture plate is then placed upon the slate, and is covered by the glass plate. The colonies

in five of the squares through which the diameter would pass, are then counted. The total is multiplied by ten, and thus the approximate number of the colonies contained in the whole plate is arrived at. Of course it is taken for granted that the colonies are pretty evenly distributed over the whole surface.

Not much, however, can be deduced from the number of the colonies alone. This may be proved by examining some distilled water, which has been allowed to

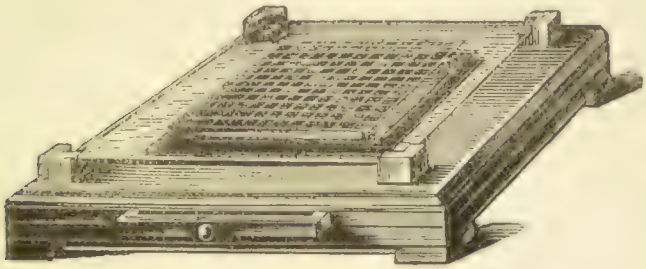

Fig. 9.

stand for some time in the laboratory, and which sometimes contains an immense number of living bacteria. If water is examined just after it has been distilled, and then again eight days later, having been allowed during the interval to stand in the laboratory at ordinary temperatures, it is seen that in the first case only isolated colonies make their appearance, whilst in the second a great number are to be seen. Thus the bacteria must have multiplied considerably during the interval, and the infinitesimal amount of foreign

matter which the distilled water contained must have sufficed for their nourishment and further development. Hence we see that the mere number of the colonies affords us no means of judging of the fitness of the water for drinking purposes; for, under these conditions we should have to condemn distilled water, which has stood for some time, as impure.

On the other hand, a great deal depends upon the *number of kinds* present. There can be no increase in the number of species present, however long the water is allowed to stand, so long as it is protected from contamination from without.

It is further to be remarked that in distilled water very few kinds—one, two, or at most three—are present, although eventually a large number of colonies appear. Also in good, pure spring water from mountains only a very few species are found, and, indeed, in consequence of its low temperature, only few individuals at all. On the other hand, if we examine water which has been contaminated by drains, we are surprised at the exceedingly great number of species that we see. Thirty or even more different kinds may be present in only a few drops; these are easily recognised by the different appearance of their colonies. This is readily explained. Impure water has come into contact with many more kinds of bacteria than pure water, and has therefore had much more opportunity of taking them up. If the temperature remains low, these bacteria

are certain to multiply but little, and thus the number of colonies, even in very impure water, may be less than in pure water, which has been kept for some time at a higher temperature. On the other hand, the number of species in both samples remains unaltered, being affected by neither time nor temperature, for during the interval which is likely to elapse, it is not probable that a species would die off and thus become extinct in the sample. Thus we see that the number of species furnishes a far more reliable criterion for forming an opinion of the water than is afforded by the number of colonies.

Finally, the *character of the species* found is of great importance. If the water is to be examined, in order to find out whether it is fit for drinking purposes, we have first to notice if there are many species present which liquefy the gelatine. These kinds are almost invariably putrefaction bacteria, in the exact sense of the word. They only thrive well where plenty of organic material suitable for their nourishment is present, being almost entirely absent in pure water. Thus they are exacting in their requirements, and are unable to multiply in pure water even at a high temperature. This may be proved by inoculating pure water with a minute quantity of such a colony; after careful mixing, some of this is poured off immediately into a culture dish, and after eight days some more into another. We then see that, whilst those bacteria

which were originally present in the distilled water have, after eight days, multiplied considerably, those which were introduced have, on the contrary, decreased in number, having been starved for want of suitable nourishment.

Thus, since great numbers of these putrefaction bacteria cannot exist in pure water, it is evident that a water which harbours many colonies of bacteria which liquefy the gelatine, must have been not only contaminated through the addition of filthy water, but must also have been so rich itself in organic nutrient substances, that the putrefaction bacteria are able to live in it.

Hence, if we wish to examine a sample in order to discover its bacteriological contents, we must take into account the number of the colonies, the number of the species, and the characteristics of these species. No great importance is to be attached to the number of colonies, as has been shown; on the other hand, water which contains a great many different kinds, amongst which are several which liquefy the gelatine, must be condemned as unfit for drinking purposes, even if the chemical analysis has been satisfactory. The question now arises, How many kinds may be allowed in drinking water?

It is evident at the outset that a hard and fast line cannot be drawn, and that our opinion must be based upon the consideration of all the facts. Above all we

must judge by numbers arrived at empirically. It may be stated that there should never be more than *ten* different species in good drinking water. It may, however, happen that a water containing fewer must be condemned, or that even if more than ten species are present, the water may be considered fit for drinking purposes.

If there are many species which liquefy the gelatine, and moreover a large number of colonies of them, the water must be considered impure, even if the number of species be less than ten. If ten or fifteen colonies which liquefy the gelatine are present in one cubic centimetre of water, the outlook is bad; if several different species are present amongst these colonies, then no doubt the water must have been in some manner contaminated. If the colonies of kinds which liquefy the gelatine are still more numerous, the water must immediately be condemned as impure.

On the other hand, it may happen that more than ten kinds of bacteria are present in water which is quite fit for drinking purposes. In these cases they are such as multiply only slowly, and do not liquefy the gelatine. These bacteria are mostly chromogenic, and probably fall into the water with the dust out of the air; they are able to exist even in the purest water, although with some difficulty. As a rule the colonies are only few.

As regards the number of colonies, a limit has been

put at 500. This is called the "fixed point." As we have seen, the number of colonies furnishes the least reliable of the data by which we have to judge, being the one most affected by outside circumstances, and therefore of the least value.

Thus the opinion formed of the water must depend, within certain fixed limits, upon individual judgment, and it is only after long practice, and after having compared many different kinds of waters, that the student is in the position to form a correct opinion.

The method of examination is somewhat different if the water is to be examined for pathogenic organisms. In by far the greatest number of cases the typhoid bacillus is the one searched for, and on this account, in addition to the ordinary examination as given above, those colonies of bacilli which resemble those of the typhoid bacillus are further investigated. There are quite a large number of bacilli, whose colonies in gelatine plates are very similar to those of the typhoid bacillus. A small portion of each suspected colony is taken with a sterilised needle, and is spread out in a drop of water upon a slide. If with the microscope the colonies still resemble those of the typhoid bacillus, portions must be inoculated on to carefully sterilised cooked potatoes, and into tubes of gelatine. It is best then to compare these cultures with similar ones prepared with typhoid bacilli: especial notice must be taken of the potato cultiva-

tions. Eventually it may be useful to stain the flagella. The characteristics of the typhoid bacillus are enumerated in detail in a later chapter. It must however be mentioned that on the other hand it is by no means easy to distinguish the typhoid bacillus from other similar organisms: in order to do so all the peculiarities must be considered together; a single characteristic, such as its growth upon potatoes, is quite insufficient by itself.

In closing this chapter it may be mentioned that in cold weather the cultivations in Esmarch's tubes are very suitable for examining the bacteria found in water. They possess this great advantage that they can be prepared and rolled out on the spot, and in many cases this is extremely useful. As a rule, however, it is more convenient to make the cultivations at home, where they can be more accurately executed.

CHAPTER XIV.

THE ORGANISMS OF SUPPURATION.

Although it has been proved that chemical compounds of various kinds, such as croton oil, turpentine, or quicksilver, if aseptically introduced into living animal tissues may produce suppuration, yet such cases as these are quite unusual, and are only produced artificially. As a general rule it may be stated that suppuration is connected with the presence of bacteria; yet apparently, however, the power of causing suppuration is not confined to one specific kind, but is common to a great number of species, the most interesting and important of which are described in the following pages, the methods of cultivating and demonstrating them being entered into with some detail. In addition to these specific suppuration bacteria there are a good number which are more rarely met with, or which only occasionally cause suppuration.

1. *Staphylococcus pyogenes aureus.*

This organism is the one most frequently met with, and apparently it is also widely distributed in nature, for it has been demonstrated several times in the dust

of the air, etc. It is present in different kinds of acute processes, and is nearly always to be found in cases of boils, of suppuration in hair follicles, and of sebaceous glands. It has also been demonstrated as the chief exciting cause of some severe diseases, such as pyæmia and ulcerative endocarditis; perhaps, or it may even be said probably, it is the cause of acute osteomyelitis, although in this case other organisms are also present. It does not often follow the course of the lymphatics, but rather destroys the tissue in circumscribed areas.

Its cultures are easily prepared, and sure to succeed. It is best, however, to take the material for inoculating the gelatine from recent abscesses, etc., and further, not from the pus itself, but from the edge of the tissue which has not yet broken down. There the micrococci have the most vitality, whereas in the pus itself now and again there are none or only dead ones to be found, and hence the cultures are a failure.

A small trace of this material is taken with a sterilised platinum wire, and is introduced into a test-tube full of fluid gelatine. After they have been well mixed together, a small portion is taken out of the test-tube and is introduced with another sterilised wire into a second test-tube full of fluid gelatine. It is scarcely necessary to make a third attenuation. The test-tubes are then emptied into sterilised glass vessels, and kept at a temperature of about 20° to 22° C.

On the second day small punctiform colonies appear, which gradually become rounded and golden-yellow, increasing in size until they are as large as pins' heads. As soon as they reach the surface of the gelatine, the latter immediately begins to liquefy, although slowly. Around the colony a very shallow depression, several millimetres in breadth, is formed; the border of this depression stands out sharply from the surrounding gelatine, although it is only to be plainly distinguished in exactly the right light. This shallow depression is caused by the liquefaction of the gelatine. By degrees the colonies increase in size until finally they coalesce and the whole of the gelatine is liquefied.

If, before the colonies run together, a portion of one is conveyed into some liquid agar-agar, and then some of the mixture is poured out on to a plate, the behaviour of the *Staphylococcus pyogenes aureus* at higher temperatures may be observed. At blood heat it grows somewhat more rapidly, and, since agar-agar is never liquefied by bacteria, and therefore it is no longer to be feared that the colonies will break down and coalesce, they may be observed for a much longer time, only of course the agar-agar must be kept moist.

Its behaviour in puncture cultivations is characteristic. At first a golden yellow film, which sinks in somewhat, appears on the surface of the gelatine, whilst along the track of the needle there is only a

delicate whitish thread to be seen. Very soon this golden film, whilst gradually increasing in size and becoming more intense in colour, sinks deeper and deeper into the slowly liquefying gelatine until after two or three weeks it has almost reached the bottom of the tube. The whitish thread is carried along too, so that finally we see, beneath the golden mass, a white one which gradually becomes yellow. The cause of this is, that the yellow colouring material can only be formed in the presence of oxygen, and therefore is absent in the thread, as sufficient air cannot gain access to the bacteria. The manner in which the gelatine is liquefied and the colonies slowly sink down, the intense orange-yellow coloration gradually merging into white, all form very characteristic peculiarities of this species, by means of which it may be easily recognised from other similar ones.

On the surface of an agar-agar puncture cultivation a yellow film appears, whilst along the track of the needle a grey or greyish-yellow thread is formed, this gradually diminishes in size as it goes downwards, but it is still to be distinctly seen, even where no air can gain access to it. Thus we see that we are dealing with a facultative anaërobic organism. If a stroke cultivation be made on agar-agar, a thick layer of a dark golden-yellow colour is formed. Similarly on cooked potatoes a golden-yellow coating is developed. Old cultures of the *Staphylococcus*

pyogenes aureus have an unpleasant smell, like sour decomposing paste.

These bacteria are pretty easily stained with most aniline dyes, but their coloration is not intense. It is therefore best to use more powerful staining solutions. Gram's method is particularly suitable, the micrococci becoming permanently stained. Faultless preparations may be obtained if the fixed cover-glass preparations are allowed to float for twenty-four hours upon the surface of a very dilute solution of fuchsine (50 ccm. of water, 10 drops of concentrated alcoholic solution of fuchsine), after which they should be thoroughly rinsed with water.

With the microscope we see irregularly arranged small roundish cells (see Plate I., Fig. 1), a little less than $\frac{1}{1000}$ mm. in diameter. If they are closely packed together, they form heaps; but if they have been very much separated, they lie either singly or in twos or threes; sometimes they are grouped as diplococci, sometimes as short three-celled threadlets, at others as three-celled pyramids, or tetrads; it never happens that chains of more than three or at most four cells are formed. In the tissues and in the pus they may occur singly or in groups, and it is in consequence of its manner of growth that it has been included in the genus *Staphylococcus*. Its manner of growth in cultivations cannot afford us any real specific characteristics, since it is so various. For

instance, if we take from an actively growing agar-agar culture a small specimen for microscopic examination, we always find a large number of diplococci, and even short three-celled chains, that is to say the commencement of streptococcus growth. True specific characters cannot be influenced by any methods of culture, they should remain constant under all conditions.

There are some other bacteria, which excite suppuration, and which are very similar to the *Staphylococcus pyogenes aureus*, but which are distinguished from it by the colour of their colonies. The most important of these are the *Staphylococcus pyogenes citreus*, and the *Staphylococcus pyogenes albus*. The former is somewhat rare, and, as a rule, only occurs in acute abscesses. Its appearance in cultivations exactly resembles that of the aureus, except for its lemon-yellow colour; neither with the microscope can any striking difference be discovered between them, only it appears as if in the citreus the cells had less inclination to group themselves together, and would rather remain isolated. The coloration of the colonies, however, is very distinctly different from the golden or orange-yellow of the *Staphylococcus pyogenes aureus*. The albus occurs more frequently than the citreus, although less frequently than the aureus; sometimes it occurs alone, sometimes in conjunction with the latter. Except that the cultures, even when old, remain white, in

its manner of slowly liquefying the gelatine, and in its development generally on plates and on agar-agar it exactly resembles the aureus. Even microscopically no difference can be observed, except that here again the cells are not so frequently arranged in groups. Lately some other very rare staphylococci have been found in pus; these also show distinct colorations, by means of which they may be distinguished from the others, but on account of the rarity of their occurrence they are practically of small importance. Whether all these species are really distinct, or whether they are only various forms of the *Staphylococcus pyogenes aureus* itself, has not been yet decided; for the present, until sufficient reasons have been given for classing them together, it is best to consider them as distinct.

2. *Streptococcus pyogenes.*

Another organism, which occurs with equal frequency as the exciting cause of suppuration, and which frequently extends itself along the lymph ducts, is the *Streptococcus pyogenes*, so-called, from its great tendency to form chains resembling strings of pearls. There are also a great number of other diseases caused by this organism, which are called by various names, and which are frequently marked by their very malignant course. Progressive gangrene, puerperal fever, and pyæmia, are very frequently, even if not invariably, caused by the *Strepto-*

coccus pyogenes. It may as well be mentioned here that no differences sufficiently important to distinguish them from one another have as yet been discovered between the streptococcus of suppuration and that of erysipelas. They are similar morphologically, and also in their behaviour in cultivations and in staining solutions. It is therefore best to class them together, especially as recent observations on their pathogenic behaviour support this view, and to consider that the slight differences which can be observed between them are caused by their mode of invasion or by physiological variations due to external conditions. We are most sure to find the *Streptococcus pyogenes* in pus which has been taken from wounds, where extensive inflammation along the course of the lymphatics has occurred. We take a small portion of this pus, and prepare plate cultures with it just as we did with the pus containing the *Staphylococcus pyogenes*. The colonies develop more slowly than those of the latter; in the interior of the gelatine they form small whitish points, scarcely as large as pins' heads, and on the surface thin colourless films. Even after having been kept for some time, the appearance of the cultures is not much changed; the colonies grow no larger, and, even after months, the gelatine has not commenced to liquefy. If a puncture cultivation is made in gelatine, and is kept at a temperature of about 22° C., after about eight days the

cultivation has developed in a characteristic way; no thread forms along the track of the needle, but in its place a number of very small completely globular colonies are to be seen, often only $\frac{1}{2}$ to $\frac{1}{4}$ mm. in thickness; they are quite isolated from one another, and even if the cultures are kept for a long time, show no inclination to coalesce. They are white, and along the whole length of the track are of approximately uniform size, thus showing that here again we are dealing with a facultative anaërobe. This peculiar mode of growth in gelatine is most characteristic of the *Streptococcus pyogenes*; there are, it is true, some other forms, which behave in a similar manner, but they are, comparatively speaking, rare, and hence of slight practical importance; we shall therefore omit to speak of them altogether. The strong likeness between the *Streptococcus* of *erysipelas*, and the *Streptococcus pyogenes*, has already been mentioned; the puncture culture of the one in gelatine is not to be distinguished from that of the other.

In a stroke cultivation of the *Streptococcus pyogenes* a thin transparent film is formed, similarly composed of small colonies, which do not readily coalesce, and which do not extend much beyond the stroke. In this case also, the organisms multiply but slightly, and in a short time the growth of the colony ceases. If the cultivations be made on agar-agar, and the temperature be raised to blood heat,

the growth is more considerable; under these circumstances the colonies unite along the stroke, forming a thin, whitish, transparent coating, which is scarcely visible at its edges. The growth upon potatoes is hardly perceptible; indeed the organisms never really develop properly. The streptococcus thrives best in bouillon, in which, in a few days, if the temperature of 37° C. be maintained, a flocculent sediment is formed, whilst the colour remains unchanged.

It may be stained in the same way as the *Staphylococcus pyogenes aureus*; it is permanently stained if treated according to Gram's method. If especially perfect preparations are required, it is well to use more dilute fuchsine solutions, and, while frequently changing this solution, and rinsing the cover-glass, to allow at least two days to the organisms to absorb the stain. In this manner preparations are produced in which the individual cells stand out especially distinctly; if stronger solutions are used, the outlines are less distinct, and the resemblance to a string of pearls is not so evident. An indistinct gelatinous substance—the swollen outer membranous covering of the cell—is apt to become stained also; and as its colour is intenser internally than externally, the cells appear to have no sharp outline, but to run into one another, thus forming a thread. Clear outlines can only be obtained if the preparations are gradually stained with very dilute solutions.

The appearance under the microscope of the *Streptococcus pyogenes* is of a chain, of varying length, consisting of globular cells arranged one after another like a string of pearls. The majority of these cells are only a little larger than those of the *Staphylococcus pyogenes,* but very frequently threads occur, in which a few single cells are distinctly larger than the rest. These larger cells are, as a rule, separated from one another by a number of smaller ones, which gives the chain the appearance of a crown of roses, although occasionally, however, two or three occur together. It is extremely probable that these are permanent forms or **Arthrospores.** The following fact, which may be observed in every very old culture, is confirmatory to this theory. In such cultivations the larger cells often are well stained, whilst the smaller ones are paler, which appears to indicate that the latter have commenced to degenerate, whilst the former are still in possession of their full vitality. The number of organisms which join together to make a chain varies considerably. Generally there are from five to ten members; chains as long as those portrayed in Fig. 3, Plate I., occur less frequently. Often the chains are coiled up into tangled balls. Sometimes, especially in liquid nutrient media, diplococci (two in a chain) occur. When division is about to take place, the cells elongate themselves, becoming constricted in their middles, whilst their ends remain

semi-circular. They are, even in this form, easily to be distinguished from the rodlets, since their cell walls are never parallel for even the smallest part of their length.

3. *Bacillus pyogenes fœtidus.*

This organism, though of rare occurrence, is shortly described here, as it is sometimes to be seen in pus. It grows very rapidly on gelatine plates; even on the second day small white spots may be observed. These quickly grow into large colonies, which may be sometimes as much as 1½ cm. in diameter, and which finally coalesce. The colonies are whitish grey in colour, and often somewhat transparent, especially towards their edges, where they are much thinner. The gelatine is not liquefied. In a puncture cultivation the bacillus spreads itself out in a similar manner on the surface, but the colony generally appears more transparent, whilst its edges are raised above the surface. All along the track of the needle the growth is insignificant, generally only very small globular colonies, sometimes irregularly confluent, sometimes isolated, are formed. On potatoes thick, slimy, yellow-brown colonies develop (the colouring matter is not always uniform, it is more or less brown or yellow according to the reaction of the potato). The growth upon agar-agar is similar to that upon gelatine, only more vigorous, and the colonies, especially as they grow older, become of a dirty brownish colour.

Staining is easily done with any aniline dye, but is especially successful when a solution of fuchsine is used. The bacilli are then seen with the microscope to be rodlets, about $\frac{1}{5000}$ mm. in length and about $\frac{1}{2000}$ mm. in breadth, with distinctly rounded-off ends. Two to four often hang together, but the separate cells are clearly marked off one from another; only rarely, and then invariably in fluid nutrient media, are large collections formed. All its cultivations have a most unpleasant smell, like decomposing egg albumen.

4. *Bacillus pyocyaneus.*

The greenish-blue coloration often present in pus and the dressings of suppurative wounds is caused by the action of a colouring matter formed by the *bacillus pyocyaneus*, an organism which until the last few years has been looked upon as a harmless inhabitant of dressings. Recent observations, however, show that it is not so innocent, but that in addition to thriving well in the pus, caused by other organisms, it itself possesses the power of exciting suppuration.

It may be very easily obtained from pus, and then used for plate cultivations. Even on the second day small turbid spots are seen in the gelatine, and soon afterwards a greyish-white point may be observed in the centre, from which delicate threads stretch out into the medium. The appearance of the colony keeps changing; at first brighter rings are formed

round the centre, and then liquefaction of the gelatine gradually takes place, starting with the first of these rings, and by degrees reaching the somewhat indistinct edges of the colony. At the same time a deep ring of gelatine around each colony becomes coloured bluish-green, being at first only slightly fluorescent, but by degrees becoming somewhat more intensely so. In puncture cultivations the gelatine is quickly liquefied, and coloured bluish green. By degrees the greenish-blue colour becomes converted into a dirty brownish green, which in its turn becomes finally quite brown. On agar-agar rather dark, dirty white, slimy films are formed, whilst the nutrient medium becomes gradually of an intense blue colour. In this case also the originally pretty bright colour becomes in time changed into an indeterminate brown. In cultivations on potatoes, when these latter are either alkaline in reaction, or are made so, the colonies are whitish brown, whilst the medium itself is distinctly green in colour. If the potatoes have an acid reaction they are only faintly brownish in colour; if after the removal of the colonies they are rendered alkaline, they immediately become distinctly and intensely bluish green. In milk this bacillus produces a dirty though bright green coloration, which is easily to be distinguished from that caused by the *bacillus cyanogenus*.

It has no special peculiarities in its behaviour towards staining reagents, being coloured with any of

the aniline dyes. In order to stain its flagella it is best to use part of a culture on agar-agar which has been kept at a temperature of 37°, and which is ten days old. In addition to the mordant described on p. 138, it is well to use five drops of the sulphuric acid, which is neutralized by the one per cent. solution of caustic soda.

The *Bacillus pyocyaneus* consists of very small slender rodlets, occurring either alone, or united together in short threads, which are composed of only a few cells. Their length is always greater than their breadth, and they may easily be distinguished from micrococci by the fact that their longer cell-walls, for some short distance at any rate, are parallel. On an average they are a little more than $\frac{1}{1000}$ mm. long, and $\frac{1}{3000}$ to $\frac{1}{4000}$ mm. broad. They are exceedingly motile and active, and, as may be demonstrated if they are treated according to Löffler's method for staining flagella, to possess one, or more rarely two flagella at one or both poles; these flagella are rather long and thin.

Many cells are swollen somewhat in the middle, and at these points show more marked refractive powers; it has not yet been worked out, whether this appearance is due to the formation of spores, or to the degeneration of the cells, the extreme minuteness of the organisms rendering observation of them by no means simple.

Two forms of this bacillus have been distinguished, one of which produces a blue colouring matter, pyocyanin, and the other a yellow-brown one. In addition, quite a large number of small differences have been observed, but these appear to be so unimportant that a division into two species does not seem to be justified. It is quite possible that the different methods of cultivation play an important part in bringing about these variations.

This organism can hardly be confused with any other; the fluorescent bacilli never cause such an intense coloration as the *Bacillus pyocyaneus*, and the bacillus of blue milk (*Bacillus cyanogenus*) does not liquefy the gelatine, and, moreover, the colour produced by it is a more steely blue, or brown, which never approaches a green.

5. *Micrococcus gonorrhœæ*. The material for examining this organism, gonorrhœal pus, is easily obtained from any hospital. As the cultivation of this micrococcus is most difficult, and can only be accomplished in human blood serum, it is best not to attempt it. We will therefore devote our attention to observing its appearance in gonorrhœal pus.

A small quantity of the pus is spread out as evenly and thinly as possible, without too much rubbing, upon some cover-glasses, and is then left to dry thoroughly in the air. The preparations are then fixed and stained. The most suitable staining solution is a

dilute aqueous solution of methylene blue, or a very dilute one of carbolic methylene blue. According to the strength of the staining reagent, the staining may take from ten minutes to several hours; as before, the best preparations are often those for which weak solutions have been used for a long time.

If the cover-glass is now examined with the microscope, amongst the pus corpuscles, some are seen which contain the *Micrococcus gonorrhœæ*, or *gonococcus*. Sometimes there are a great many together, sometimes they are more isolated. Only exceptionally is this organism found outside of the corpuscles, and then only in consequence of the cells having been crushed up one against the other during the preparation of the cover-glass, so that they have been ruptured. The plasma becomes diffused, but the nuclei remain intact, and around these the micrococci are grouped, distinctly separated one from the other. Such a cell has been selected for representation in Fig. 2, Plate I., because in this condition the peculiar biscuit-shaped form of the gonococcus is more easily to be seen than when it lies enclosed in a cell. Gonococci, as a rule, are grouped together in pairs, but they do not touch each other, being distinctly separated from each other. They are not quite globular in form, but on the outer side are semicircular, and on the inner side somewhat flattened. More rarely isolated micrococci are to be seen, but

of these even the greater number are really diplococci, of which only one cell can be seen, for as their long axes are perpendicular to the surface of the slide, they lie one above the other. They vary very much in size; on an average their longest diameter is from $\frac{1}{1000}$ to $\frac{1}{2000}$ of a mm. in length.

By its characteristic form, and also by its peculiar position, either in or upon the cell, the gonococcus may be distinguished from all other similar organisms.

After treatment with the methylene blue, as described above, the gonococci themselves should be coloured an intense blue, whilst the nuclei are paler, and the plasma is only faintly tinted. Gram's method must not be used, as the micrococci become immediately decolourised. Moreover, the preparations must only be rinsed in water, or at the most should be dipped for a moment in one per cent. acetic acid, and then immediately rinsed with distilled water in order to prevent the slight decolourisation which has by this means been produced in the pus corpuscles from spreading to the gonococci. Contrast staining may also be attempted; the preparation is first coloured with eosin, after which it is treated with methylene blue; by this means the pus corpuscles are coloured red, and the gonococci blue.

These preparations, however, are not distinct in outline, and are therefore not to be recommended.

If it is desirable to bring out the gonococci still more distinctly, it is well to treat the preparation beforehand with acetic acid. If the beauty of the preparation is not to be considered, but it is only wished to stain the gonococci quickly, the cover-glass need only be kept for two or three minutes in concentrated aqueous fuchsine solution. The gonococci are very intensely stained, and stand out quite clearly enough from the elements of the pus, which are also, to be sure, intensely coloured themselves.

CHAPTER XV.

THE ANTHRAX BACILLUS (BACILLUS ANTHRACIS).

The anthrax bacillus is not only pathogenic in a great many animals, but also in man, in whom it causes the well-known malignant pustule. Amongst animals it occurs most frequently in cattle, in which it only exceedingly rarely remains localised to one spot, but on the contrary spreads itself throughout the whole body, occurring chiefly in the blood vessels. In consequence, these bacilli are to be found in great numbers in the blood of animals which are ill or have died of this disease,

If a drop of blood from an infected animal, which is either still alive or has only just died, be placed upon a slide and be examined with a moderately high power ($\times 300$–500) of the microscope we find innumerable small glass-like rodlets distributed amongst the blood corpuscles.

These are always non-motile, and are from $\frac{5}{1000}$ to $\frac{80}{1000}$ mm. long, and a little over $\frac{1}{1000}$ mm. broad. If the blood sample is not taken until some time after the death of the animal, the rodlets are seen to have

developed into longer or shorter threads, which, as a rule, are much entangled and twisted together. Small portions of the blood can be spread upon cover-glasses for future staining.

In order to prepare a pure culture, we proceed in the usual manner. A small portion of blood is well mixed in a test-tube with some fluid gelatine. From this one a second, and from the second a third tube full of gelatine is inoculated in the usual way. The contents of the third test-tube are then poured out on to plates, which, after the gelatine has solidified, are placed in an incubator, which is kept at a temperature of about 20° C.

On the next day, very small whitish points are visible with the naked eye. By the day after that these points have developed into whitish spots, of about one millimetre in diameter, the outlines of which are indistinct and wavy, becoming imperceptibly merged in the gelatine.

Even with the naked eye it can be seen that the colonies are not quite homogeneous. If one of them be examined with a low power of the microscope ($\times$ 50–80) a most characteristic appearance immediately presents itself by means of which the anthrax bacillus can be both easily and surely distinguished from all other kinds of bacteria. Peculiar wavy strings of bacterium threads can be seen twisted backwards and forwards in every direction. No isolated bacilli

are found, only these long threads, which are twisted together in wavy strands. These strands are sometimes quite close together, and sometimes further apart, so that the appearance presented by the colony has been correctly compared with a mass of curly hair; like natural locks on a curly head, the strands of bacillus threads proceed from the centre to the margin, becoming gradually less dense and entangled as they approach the edge of the mass.

Liquefaction of the gelatine gradually takes place, commencing in the centre and spreading towards the margin. As the gelatine becomes liquid the colony loses its characteristic appearance, until after a time this is only to be seen at the edges, where the medium is still solid.

Before the area of liquid gelatine is large, and before the colonies exceed one or two millimetres in diameter, an impression cover-slip preparation should be made in order to preserve this characteristic growth.

A thoroughly clean cover-glass is taken up with the forceps, and so placed that one edge rests on the gelatine, whilst the rest is held in a slanting direction over a colony; the glass is then allowed to sink gradually down, care being taken to allow the air which is between it and the somewhat sunken centre of the colony to escape. It is hardly necessary to press the cover-glass down; it is better to lift it up

with the forceps as quickly as possible, with the colony adhering to it. Such a preparation is called an "impression cover-glass preparation." It should be then put aside for a time to dry.

In order to observe the different ways in which the anthrax bacillus grows on different media, test-tubes containing gelatine and agar-agar are inoculated with small portions taken from one of the colonies of a plate culture. Its growth on potatoes is not peculiar; dull, greyish-white masses, generally only a few millimetres in breadth, develop along the track of the needle. On agar-agar a slimy film is formed, in colour of a dirty white, often inclining to red; after a few days, peculiar bright spots appear, through which the agar-agar can be seen, so that it looks as though bubbles had been formed at these places. The gelatine soon becomes liquefied; this occurs least rapidly in puncture cultivations and only after another equally characteristic growth has shown itself. At first a thin white thread appears along the track of the needle. This becomes thicker and thicker the nearer it approaches the surface, whilst it gradually disappears in the lower layers of the gelatine. A great number of fine threads radiate out from the central one, and, as a rule, become branched again. These diminish in number as they descend, so that at the end of the thread there are scarcely any to be seen. On the surface they sometimes extend right

up to the sides of the test-tube. At the same time the gelatine in the neighbourhood of the puncture liquefies, the liquid gelatine gradually spreading downwards and also superficially, until the sides of the test-tube are reached. After ten days, as a rule, the layer of liquid gelatine is about two or three centimetres in depth, and the characteristic appearance of the puncture-culture has disappeared, for the colonies now look like a whitish flocculent precipitate resting upon that portion of the gelatine which is still solid. Generally, growth is arrested by this time, for the layer of liquid gelatine prevents the bacilli from obtaining the oxygen which is absolutely necessary for their development. As a rule, the liquefaction of the gelatine is also arrested; only in very rare cases does it proceed any farther, and then only very slowly.

The anthrax bacillus also forms spores. In order to observe this process more closely, it is best to make a cover-glass cultivation in the hanging drop. A cover-glass and an excavated slide are sterilised in the usual fashion, and upon the former a drop of sterilised nutrient agar-agar is placed. When this has solidified, a small portion of a plate cultivation colony is transferred to it with the platinum needle. The cover-glass is then reversed, and placed over the slide with the drop hanging down; the whole affair is then closed up with a drop of paraffin oil, and placed in an

incubator, which is kept at a temperature of 20°. The bacteria rapidly grow into threads, but only commence to form spores after they have exhausted the nourishment, a condition of things which arises in a longer or shorter time, according to the amount of agar-agar and the number of bacilli present.

But spores can only be formed if a sufficient quantity of air has been enclosed in the cavity of the slide, as they require an ample supply of oxygen in order to develop.

If necessary, the cover-glass may be raised with a needle, in order to allow fresh air to enter, but it is better at the first to choose a slide with a very large cavity or one which has a glass cell cemented on to it.

The first indication of the commencement of the formation of spores is, that the threads become less distinct and lose their great refractive power, whilst fine granules make their appearance inside them. Sometimes the cell walls appear to stand out more clearly, but frequently they remain invisible. In each cell a small bright point appears, which gradually increases in size, whilst becoming brighter and more refractive, so that it appears to shine out from the faintly grey thread. Finally, it reaches the cell wall, when it grows oval in shape, and at the same time becomes clearly marked off by a sharp outline from the surrounding protoplasm. As this body becomes brighter and brighter the rodlet on the other hand

becomes more and more indistinct until at last the threads fall to pieces, the separate cells break up, and the bright elliptical corpuscles, the spores, are set free.

At first no further changes occur in the spores; if however a few are introduced with a needle into a drop of bouillon or agar-agar upon a cover-glass, and are then cultivated in an incubator, in a few hours changes take place. At one or other pole of the spore the very tender spore membrane breaks down, thus allowing the budding rodlet to emerge. This rodlet grows longer **in the direction of the length of the spore.** At the same time the highly refractive body, which at first completely filled the interior of the spore membrane, becomes gradually smaller and smaller, until finally, when the rodlet is full grown, it has entirely disappeared. The empty membrane generally remains, attached like a delicate veil to one end of the rodlet, but at last it drops off and falls to pieces. The young rodlet soon begins to divide, and growth proceeds rapidly until all the nourishment is exhausted, when spores again begin to be formed in the thread.

The observation of this germination of the spores affords us an infallible means of distinguishing the anthrax bacillus from the hay bacillus, with which formerly it was frequently confused; in the latter the budding rodlet breaks through the side of the spore, in the former it emerges from one or other pole. In

addition, there are numerous other differences to be observed between them; the anthrax bacillus is thicker than the hay bacillus, and forms longer rodlets; it is always non-motile, whilst the hay bacillus always passes through motile stages, especially after budding has taken place. In addition, the appearance of their colonies in the various cultures is very different.

The anthrax bacillus is easily stained with any of the usual staining reagents; gentian violet gives the prettiest effect. Gram's method may be used, and with special advantage, when sections and blood preparations are to be doubly stained. If from a young agar-agar culture a small portion be taken, and a dry cover-glass preparation be made with it, the thread, after suitable staining, may be seen to hang together, although the separate cells are clearly to be distinguished. If, on the other hand, a blood preparation is stained, the threads are seen to be much shorter, and between the cells composing them greater gaps occur, which do not have parallel but lenticular walls, as the shorter walls of the cells are concave. The impression cover-glass preparations taken from plate cultures must be well dried before they are fixed. When they are *cold* they may then be carefully stained. If the staining solution is heated, the colonies, especially if they are a little old, become easily loosened from the cover-glass. Spores may be easily rendered visible if

treated according to the method of contrast-staining described on p. 114; the effect is especially distinct, if the spores are stained with aniline water solution of fuchsine and the threads slightly with methylene blue. (cf. Fig. 4, Plate I.). In sections it will be seen that the bacilli occur in the blood capillaries, which are often completely blocked by them. The liver, spleen, and kidneys are especially infested, and in them large numbers are always to be found.

In all these experiments with the anthrax bacillus, and indeed with all pathogenic bacteria, it is necessary to take the very greatest care to avoid engendering the disease in oneself or in others.

CHAPTER XVI.

BACILLI OF CHAUBERT'S DISEASE (RAUSCHBRAND), OF MALIGNANT ŒDEMA AND OF TETANUS.

In isolated districts in many countries, a disease occurs amongst cattle, which for a long time was confused with anthrax. When however it was proved by means of the examination of sections that the two diseases were distinct, search was made for the exciting cause of the former; this was finally discovered to be a strongly anaërobic bacillus. The disease has acquired its name of "*Rauschbrand*," from the fact that peculiar swellings caused by collections of gas occur in various parts of the body, especially on the breast and thighs, and that these swellings crepitate when they are stroked by the hand.

If we examine a drop of the exudation from such a place with the microscope, we find bacilli which on an average are from ·004 to ·006 millimetres long and about one-eighth as thick, and which occur either singly or connected together in pairs. The ordinary methods of cultivation do not answer with this bacterium, as it is strongly anaërobic. It is therefore

necessary to employ the method described in Chapter V., and in order to obtain a pure culture to prepare with a small trace of the exudation an Esmarch's tube in an atmosphere of hydrogen, and to keep it in an incubator at a temperature of from 20° to 22° C. After two or three days white colonies, which liquefy the gelatine rapidly, make their appearance. If some strong grape-sugar gelatine be placed in a test-tube and be inoculated with a portion of one of these colonies, the anaërobic nature of these bacilli very soon becomes apparent. They flourish even at ordinary temperatures, and liquefy the gelatine very energetically. This liquefaction, if too many spores have not been transferred, commences about a thumb's thickness below the surface, being caused by small isolated colonies, which are formed along the track of the needles and which soon coalesce; it progresses however very rapidly, so that very soon a thick whitish column of liquid indicates the path of the needle. Bubbles of gas, which at first are kept under the solid surface of the gelatine, are formed, whilst the liquefaction continues to spread rapidly until the sides of the test-tube are reached. Finally even the solid upper portion of the gelatine is liquefied by the action of ferments produced by the bacteria, and then the bubbles of gas rise to the surface of the medium, which is now entirely fluid. Very peculiar-looking cultures are obtained by thoroughly mixing a small

quantity of a Chaubert's disease colony with some liquid gelatine in a test-tube. The upper part of the gelatine remains sterile to a depth of two or three centimetres, whilst the lower portion becomes studded with small globules, filled with a whitish liquid, in which at first a denser nodule may be distinguished. Soon, however, gas-bubbles appear distributed throughout the gelatine; these alter the appearance of the culture, and break up the medium. In agar-agar a cloudy white thread is developed, which, having no distinct outlines, becomes imperceptibly merged in the medium; the gas-bubbles appear so soon in this medium that no characteristic appearance can be formed. Occasionally such a violent formation of gas occurs, that part of the agar-agar is lifted right up and is forced, with the plug of cotton-wool, right out of the tube; this is not of rare occurrence if the culture is kept at blood heat. If a small quantity of a colony of this bacillus taken from an Esmarch's tube be examined under water with a moderately high power of the microscope, a distinct movement may be observed in some of the cells. The organs of motion may be rendered visible, but the bacilli which are swimming about in the fluid gelatine are not suitable for this purpose, as too much gelatine is transferred with them into the preparation; this gelatine becomes stained as well as the flagella so that it is hard to distinguish them from one another. It is best to

inoculate some agar-agar, which has recently been well boiled and then allowed to solidify in a slanting position, and then to replace the air with hydrogen in the manner described on p. 73. The tubes are then carefully closed and are kept at blood heat in an incubator. Then after from five to eight hours dry cover-glass preparations can be prepared from them. Some of these may be immediately stained with aqueous aniline dyes, and thoroughly rinsed with water, whilst others are put aside in order that the flagella may be stained on a future occasion. These bacilli may be easily stained, the preparations being especially successful when fuchsine is used; however, when they are treated by Gram's method, the results are unsatisfactory.

In order to stain the flagella, thirty-five drops of the caustic soda solution are added to the mordant, and the experiment is conducted in the manner described in Chapter XI.; there are very numerous flagella, distributed all over the body of this bacillus. Occasionally, but not frequently, a peculiar formation like a plait may be observed. This is often thicker than the bacteria, and becomes more intensely stained. It probably consists of a very great number of flagella twisted up together; this seems especially probable, as such a formation very often appears to get unwound at one extremity, so that it terminates in a number of small ends. As a rule these formations are

isolated, but occasionally by good fortune one may be found attached to a bacillus.

Under favourable conditions, and especially quickly at blood heat, the Rauschbrand bacillus forms spores of an elliptical shape. They lie towards one extremity of the rodlet, and are thicker than it, so that one part, a short distance from the end, becomes swollen. As a rule mature spores are found on the third day in cultures that have been kept warm. The spores require to be doubly stained in order to be rendered visible, the result in this case is specially satisfactory, as the spores are so large.

An organism, very similar in its behaviour towards oxygen, is the bacillus of malignant œdema (*Bacillus œdematis maligni*) which is distributed everywhere in nature, being present in dung, in manured earth, in several putrifying liquids, etc. It is pathogenic in many very different organisms, but it appears only to occur in man, when through other circumstances the system has been weakened.

This bacillus can be fairly certainly obtained if samples of garden mould taken from the surface of various places, down to about 1½ centimetres in depth, are well mixed together, and cultivations are made either directly with some of the mixture, or from the liquid in which it has been soaked. It is best, as before, to prepare Esmarch tube cultures in an atmosphere of hydrogen, and to keep them at a temperature

of about 20° C. As a rule several different kinds of anaërobes develop, which it is very difficult to distinguish from one another, and therefore a puncture cultivation in strong grape-sugar gelatine is made with a portion of each colony. In this manner probably a large number of different anaërobic bacteria will be obtained, and amongst others doubtless the bacillus of malignant œdema.

This bacillus is strongly anaërobic; in a puncture cultivation it only develops at a depth of two fingers breadth below the surface. Its appearance in such a culture is not dissimilar to that of the Rauschbrand bacillus; the gelatine is energetically liquefied, and gas bubbles are produced. Even when examined with the microscope they still appear similar, only the bacilli of malignant œdema are more slender, and are shortly rounded off at their ends; moreover, they never form such regular threads as the Rauschbrand bacilli, but as a rule no more than six to ten cells join together in strings. They are readily and intensely stained with any aqueous aniline dye, but the preparations are spoiled if treated according to Gram's method. The œdema bacillus is distinctly motile, but it is not very active; its organs of movement are distributed all over its body. In order to demonstrate them it is necessary to add thirty-six drops of caustic soda solution to the mordant, and for the rest to treat the preparations like those of the Rauschbrand bacillus.

Under favourable circumstances, especially if the temperature is sufficiently high, spores are formed. These are elliptical in form, do not lie exactly in the centre of the cell, and are very resistant. Generally the cells are distinctly swollen in the middle. If once the spores are allowed to become fully mature, contrast staining is no longer very successful, as the rodlets themselves no longer become distinctly coloured.

A third very interesting anaërobe is the **Tetanus bacillus.** Tetanus, one of the most terrible of diseases, occurs in man, and almost without exception ends fatally. It has been observed that this illness occurs most frequently as a consequence of wounds to which earth, especially such as contains decomposing organic matter, has come into contact, even if the injury were only an imperceptible tearing of the skin, or a mere scratch. In such wounds a delicate slender bacillus, at one end of which there is frequently a spore, has been found. This spore is much thicker than the bacillus itself, and thus causes a great swelling in it, so that a peculiar drum-stick appearance is produced, which is very characteristic.

The *Tetanus bacillus* can only be cultivated if the air is completely excluded ; it is quite as sensitive to the presence of oxygen as the two preceding bacteria. It develops best at blood heat, but growth is not arrested, although it is not so rapid, if the preparations are kept at ordinary room temperatures. In

gelatine a feathery cloudiness is developed along the track of the needle, this spreads far into the gelatine, which becomes gradually liquefied. A similar appearance is to be seen in agar-agar, but as this medium is never so transparent as gelatine, it cannot be so clearly distinguished.

The *Tetanus bacillus* is easily stained; even by Gram's method.

The greatest care must be taken in all experiments with this bacillus; all vessels, etc., which are used should be carefully sterilised before they are put away.

CHAPTER XVII.

THE TYPHOID BACILLUS.

The typhoid bacillus plays a most important part in a very large number of bacteriological examinations of water, and it is therefore of great importance to learn to recognise it with certainty. This, however, is a not very simple matter, as the characteristics of this bacillus are very indefinite.

Material may be probably procured in the following manner, if a pure culture is not to be obtained. A small portion of fresh excreta from a patient, in a tolerably early stage of the disease, is obtained from a hospital, and with it plate cultivations are made. Typhoid bacilli cannot always be secured in this manner, but in certain stages of the disease, they are often present in great numbers in the excreta, in which case they are certain to appear on the plate cultures. Ordinary nutrient gelatine is used, and the temperature should be kept at about 20° C.

If, however, a pure culture of the typhoid bacillus is to be procured, this may be used as the original material for further investigations. The first thing to do is to prepare plate cultures, in order to learn the

characteristic growth of the typhoid bacillus upon gelatine. These plates are then put into the incubator and kept at a temperature of from 20° to 22° C. On the second day the development of the colonies has proceeded so far that they can be seen with the naked eye; twenty-four hours later it can be further seen that they are not all alike. Some have spread themselves out on the surface, forming a translucent, almost transparent mass, which lies on the gelatine like a delicate coating with uneven edges. From the centre of such a colony there are radiating lines some of which reach to the circumference, whilst others stop half way; sometimes again they start midway between the centre and the periphery, and stretch up to the latter. In a similar manner there are lines which run parallel to the circumference, sometimes right round the colony, and sometimes only for part of the way; these always keep the direction, but like the radiating lines are without a fixed arrangement. Thus the colonies have a most peculiar appearance. Gradually they grow larger and larger, and by degrees lose at their centres this characteristic appearance, which however is still to be seen at the edges. Many minerals exhibit a similar structure at the point of fracture, which is then described as a conchoidal fracture. These superficial colonies, under favourable circumstances, especially when they are only a few on each plate, become more than 1 cm. in diameter.

On the other hand, the behaviour of those colonies which remain in the interior of the gelatine, and which even after a long time do not reach the surface, is very different. They always remain small, being only about 1 mm. in length, and from $\frac{1}{2}$ to $\frac{3}{4}$ mm. in breadth; they are in shape like a lemon or whetstone, having generally clearly marked poles, and are opaque and yellowish grey in colour.

If a small quantity of one of these colonies be transferred with a sterilised platinum wire to some gelatine or agar-agar, which has solidified in a slanting direction, a thick glistening greyish white coating of the consistency of mucus is developed along the stroke, spreading out somewhat from it on either side. If a puncture is made, only a slender white thread is formed along the track of the needle, whilst on the surface a similar coating is formed as in the stroke cultivation. The gelatine is never liquefied.

On the other hand, the potato cultivations are very characteristic, and by means of them otherwise doubtful samples may often be recognised. If a potato culture is prepared in the manner described in Chapter II., and then a platinum needle, which has been first sterilised and then charged with typhoid bacilli, is drawn across it, the bacilli develop with great vigour at blood heat. At first, however, this growth is not perceived; it is only when a scratch is made on the slice of potato that the operator sees that the whole

surface is covered with a thick almost felt-like coating, which consists of a very vigorous growth of the colonies of the bacteria. Unfortunately these bacilli appear to be capricious in their behaviour on potato slices, for they do not always show this characteristic appearance. Either the colonies remain confined to the inoculating stroke, without spreading all over the surface, or the growth is to be perceived, appearing as a whitish, or slightly yellowish grey film, which may be seen without disturbing the surface. It is supposed that this atypical growth of the typhoid bacillus is due to the acid reaction of some potatoes, and indeed, as a rule, the characteristic appearance can be made to manifest itself if the potato is rendered alkaline by the use of weak soda solution. This, however, is not always sufficient, and thus the phenomena cannot be fully explained. However, these cases of atypical growth of the typhoid bacillus on the potato are not of very frequent occurrence, so that its cultivation on potato may be considered as affording the surest means for its recognition.

If samples are taken from various young cultures, and are examined in the hanging drop, it is immediately noticed that the bacilli are exceedingly motile. There is nothing characteristic either in the movement or in the form of these bacilli. As a rule they are thick rodlets, of from $\frac{2}{1000}$ to $\frac{3}{1000}$ mm. in length, with rounded ends. Occasionally two or more

unite to form short threads, which, in an unstained preparation, look like a single rodlet. In older cultures, much longer rodlets are seen, which, even when stained, appear to consist of a single cell. On potatoes often somewhat bent rods are formed, especially if the potatoes have an acid reaction and the cultures are old.

So-called polar granules are found in potato cultures, and occasionally in those on other nutrient media. These are collections of protoplasm at the ends of the cells, which become more intensely stained than the remaining plasma. In addition to these granules, there are usually places in which there is no plasma, and which are of course unaffected when the preparation is stained. Formerly it was considered that these granules were spores, but it is more likely that they indicate the commencement of degeneration in the cells. At present the formation of spores by these bacilli has not been observed. These granules may pretty certainly be always obtained from very acid or very alkaline potato cultivations, and they may be considered to constitute a reliable specific characteristic, by means of which to distinguish the typhoid bacilli from many other similar organisms.

Typhoid bacilli are fairly easily stained although not intensely with any of the usual aniline dyes. In order to obtain a well coloured preparation it is best to use an aqueous solution of gentian violet, and to

stain the dry cover-glass preparation at a moderate heat. Good results are also obtained if the cover-glass is kept for twenty-four hours in cold carbolic fuchsine solution which has been diluted with three parts of water.

The flagella of the typhoid bacilli can be very beautifully stained, if to the ordinary mordant exactly 1% of caustic soda solution be added. Great care must be taken in measuring this quantity, as the flagella are very much affected even by slight variations in this respect. They are very long, and there are great numbers of them all over the body. (Fig. 8, Plate II.). It is not easy to render them visible. It is best for this purpose to use agar-agar stroke cultures, which are only five hours old, and which have been kept at blood heat in an incubator. It is true that where the cultures are older, the flagella are more easily stained, but then, on the other hand, they are frequently thrown off from the bacteria, when they are seen to be dispersed irregularly in great numbers throughout the preparation. For further details see Chapter XI.

Neither is it quite easy to demonstrate this bacillus in the human body. It is best for this purpose to make thin sections of the intestine. When considerable numbers of bacilli are present, which is not the case in every stage of the disease, they will be seen to occur either collected together in small clumps

or irregularly distributed throughout the tissue, so that in nearly every section some will be seen. It is distinctly more difficult to find the bacilli in other organs, such as in the liver, the kidneys, or the spleen; for although they occur generally in great numbers in the spleen, they are not distributed throughout the organ, but are collected together in large deposits, which sometimes contain hundreds, or even thousands of them, whilst other parts remain quite free, so that very often a very great many sections may be cut in which not a single bacillus can be seen.[1]

It is especially difficult to demonstrate the presence of the typhoid bacillus in drinking water, and, considering the number of experiments that have been made, has been very rarely done. The methods for ex-

[1] An intense coloration may be produced by the following method, which is recommended by Nicolle, in the *Annales de l'Institut Pasteur* of Nov. 25th, 1892. Sections which have been dehydrated by means of alcohol are immersed for from one to three minutes in a solution of Löffler's or Kuhne's methylene blue. After having been rinsed with water, they are treated with a ten per cent. solution of tannin, the action of which is almost instantaneous. They are next rinsed with water, dehydrated with absolute alcohol, and cleared with oil of cloves or of bergamot. By this means the micro-organisms are stained an intense blue, whilst the tissue is not over-coloured. Excellent results have thus been obtained with sections containing bacilli of typhoid, glanders, hog-cholera, chicken cholera, and other bacteria which it is difficult to stain intensely.

amining water bacteriologically have been entered into fully in Chapter XIII.; it is not therefore necessary to describe them here. Now and again in specimens of suspected water, of which plate cultivations have been made, we may come across colonies, which, especially in their lemon-shaped appearance, have a great resemblance to those of the typhoid bacillus. Under all circumstances it is absolutely necessary to make potato cultures of these bacteria. It may also sometimes happen that milk is suspected of harbouring typhoid bacilli, since widespread epidemics have occurred, especially in Holland, which could only have been caused by infected milk. The examination of milk is essentially the same as that of water, only in this case very small quantities must be used for the cultivations, since milk, even if it has only stood for a few hours, harbours enormous quantities of bacteria, at any rate, in warm weather.

CHAPTER XVIII.

THE TUBERCLE BACILLUS.

The tubercle bacillus is to be distinguished from all others, with the exception of the leprosy bacillus, by its peculiar behaviour towards staining reagents. It is only imperfectly stained with the ordinary solutions, even when they are used for a considerable time. The best results are obtained by using aniline water solution of fuchsine, the use of which is fully described in Chapter IX., and therefore need not be entered into here. It may, however, be mentioned that less satisfactory results are obtained if the bacilli in the tissues are treated with Ziehl's carbolic fuchsine, and it is best therefore invariably to make use of the aniline water staining solutions.

The tubercle bacillus is a very delicate, slender rodlet, from $\frac{2}{1000}$ to $\frac{6}{1000}$ mm. in length, and of minute width. The cells are hardly ever quite straight, but are generally curved or bent; they are sometimes also divided in a peculiar manner. Especially in stained preparations it has been remarked that between intensely coloured portions completely colour-

less gaps are to be seen, which formerly were considered to be spores. At present this theory has been given up by most bacteriologists, as there do not seem sufficient grounds for belief in it.

There are certain difficulties which are met with in connection with the culture of tubercle bacilli. Above all, it requires great skill and practice on the part of the experimenter to obtain a pure culture from the original material. The method to be employed is described here, although the student is not recommended to attempt to prepare pure cultures from tuberculous material, as it requires a great deal of time, and the result is only very doubtful.

First of all, some ox-blood serum must be obtained, and allowed to solidify, in a slanting direction, in wide test-tubes. It is a good plan to keep it for a day or two in the incubator before using it, so as to make sure that it is thoroughly sterilised. The next thing is to procure a tuberculous animal; as guinea-pigs are very susceptible to tuberculosis, it is, as a rule, very easy to find one that is affected, or at any rate to induce the disease in one by inoculating it with tuberculous sputum. Directly after death the animal is immersed in alcohol, which is set alight, so that, as a rule, the hair is thoroughly singed off, and the skin sterilised to a certain extent, without the tubercle bacilli in the interior of the animal being affected. The skin is now cut through with sterilised scissors

that are still warm, and the tuberculous organs taken out with sterilised knives. These organs are then cut up with instruments which are frequently being changed and freshly sterilised, so that freshly cut small portions may be continually taken up. One of these pieces is then crushed between two scalpels, or better, it is placed upon a freshly sterilised sheet of zinc, and broken up with the scalpel. A small quantity of this material is then taken up with a sterilised platinum wire, and spread over the blood serum, care being taken to disturb the surface as little as possible. The test-tubes which have been inoculated in this way are then made air-tight in the manner described before, by means of india-rubber caps, and are kept at a temperature of 37° to 37·5° C. in the incubator. The temperature must be kept as constant as possible.

After a lapse of fourteen days, the first naked eye indications of the formation of colonies can be perceived in those tubes which have remained uncontaminated with other bacteria, and which contain tubercle bacilli. Little whitish specks appear, which in time become converted into dry scales, and which rest loosely on the nutrient medium. These scales never run together, but may be lifted up one by one from the blood serum; they are very characteristic in their general appearance of tubercle bacilli, for no other kind of bacterium grows in a similar manner upon blood serum. If some blood serum which has

been allowed to solidify on cover-glasses, and which has been carefully kept in the moist chamber, is inoculated with a small portion of one of these colonies, the growth of the tubercle bacilli may be observed from time to time with the microscope. Peculiarly twisted chains of cells, like notes of interrogation, are formed, which gradually become thicker in the middle, while their pointed ends become extended along the surface of the nutrient medium. It must, however, be mentioned that, even with the greatest care, these cover-glass cultivations do not always succeed.

The bacilli grow in a similar manner on agar-agar, but the cultures are more difficult to prepare. If a small quantity (from 6 to 8 per cent.) of glycerine be added to the agar-agar, the growth is much more luxurious, but the glycerine interferes with the characteristic appearance of the colonies. The more glycerine which is added, the less dry are the scales, the more do they flow into one another, and the more extended are the colonies.

These cultures of tubercle bacilli must be most carefully protected from the action of daylight. Direct sunlight often kills the colonies outright in a few minutes; even diffused light has the same effect in a few days or weeks, according to its intensity. Otherwise the cultures are very resistant, and only require to be re-inoculated every two or three months.

They must, however, of course be kept at a temperature above 29° C., as below this their growth is entirely arrested; at blood heat it is only moderately rapid. Variations of temperature of even only tenths of degrees may exert an influence upon the rapidity of their growth. This bacillus does not thrive in gelatine, on account of the above-mentioned peculiarities; it develops in bouillon at blood heat, but its growth is not characteristic.

Exceedingly similar to this bacillus, both in its morphological characters and in its behaviour towards staining reagents, is the bacillus of fowls' tuberculosis. The form of the colonies, however, is somewhat different; and also in its pathogenic peculiarities towards certain animals it shows a not inconsiderable variation. For instance, the bacillus of fowls' tuberculosis does not form small dry scales on blood serum, but a more even, mucoid, whitish coating; moreover, these bacilli, as a rule, are not pathogenic in guinea-pigs, which are so exceedingly susceptible to tuberculosis.

Bacilli were first discovered in leprous patients a long time ago, being collected together in great masses in the tissue cells of the leprosy nodules. These bacilli are exceedingly similar to those of tuberculosis, only they are generally somewhat shorter and thicker. In their behaviour towards staining reagents they also resemble tubercle bacilli, except that

they are easily stained with the aqueous solution of fuchsine at ordinary temperatures. It is almost as difficult to decolourise them, and thus by these characteristics they may be distinguished on the one hand from tubercle bacilli, and on the other from all other bacteria. It appears that a completely successful culture of the leprosy bacillus has only once been obtained, and even in this case such varying forms appeared on the peptone glycerine blood serum, that it seems as though only those bacilli which had been affected by unfavourable conditions (involution forms) could have been present. Further, inoculation of animals up to now has never succeeded in a manner quite free from objections.

CHAPTER XIX.

PATHOGENIC SPIRILLA.

The cholera bacillus, cholera vibrio, comma bacillus, or whatever else it may be called, was discovered to be present in all cases of Asiatic cholera by Robert Koch in 1883, during his journey of scientific discovery through Egypt and India. Since, as a rule, there is no opportunity of obtaining this bacillus from the excreta of cholera patients, we are forced to use pure cultures, which may be had from any of the sources mentioned in the introduction. It stands to reason that the very greatest care must be taken in handling these bacilli, so as to run as little risk as possible of engendering this frightful disease in ourselves or in others. On the other hand, it is necessary to learn the specific characters of this organism very thoroughly, so that in case of the approach of a cholera epidemic we may be able to notice and recognise it with absolute certainty.

The comma bacillus is a spirillum. It occurs most commonly in the form of short, slightly bent rodlets; hence its name of comma bacillus. In this form its

curve is about one-third of a single turn of a spiral. In other cases, especially in rather old bouillon cultures, long screw-like threads, having three, five, ten, or even more turns, develop. These constitute the spirillum form of the comma bacillus. The rodlets are more or less curved, with rounded off ends. It is true that they often, when examined with the microscope, appear to be straight, but this is when they are lying, as it were, on their dorsal or ventral surfaces. They are often very actively motile, especially in recent cultivations; and by Löffler's method of staining flagella, their motile organs have been successfully demonstrated. These are placed either singly or in pairs (very rarely in threes) at one or both poles of the cells. The statement that each cell possesses only one polar flagellum is not quite correct; even those authors which make this statement show in the photographs accompanying their writings that the cholera bacillus occasionally possesses two polar flagella.

These bacilli, as a rule, are easily stained, but they do not generally become intensely coloured; the bacteria seem sometimes, without any apparent reason, to have more affinity for the staining reagents than at others. If strongly coloured preparations are desired it is best to leave the fixed cover-glasses for at least twelve hours in a cold solution of carbolic fuchsine (1 of carbolic fuchsine to 3 of water). Such preparations on account of their intense coloration, are especially

suitable for photographing; the ones stained with ordinary aqueous solutions of aniline dyes are too pale, whilst powerful staining reagents, such as pure carbolic fuchsine, and aniline water gentian violet affect the shape of the bacilli. Similarly Gram's method cannot be employed, for the bacteria become changed in form as well as decolourised. In order to demonstrate the flagella, it is necessary to add to the ordinary mordant one drop of the sulphuric acid which is expressly prepared for this purpose.

The cultivation of the comma bacillus succeeds on all the ordinary nutrient media at ordinary temperatures, but especially well at blood heat. Upon gelatine plates kept at a temperature of about 20° C., small white flecks appear, which have not quite clear outlines, and which when examined with a low power show variously shaped out-growths and slight elevations on their surfaces. Gradually the colonies grow into small greyish-yellow masses, whilst the gelatine becomes slowly liquefied. In puncture cultivations a thin white thread appears after a few days along the track of the needle. This thread suddenly widens out just below the surface, causing a distinct liquefaction and disappearance of the gelatine at this place, whilst a bright glistening bubble of air is drawn into the liquefaction funnel. The whitish thread along the track of the needle broadens somewhat, but never becomes of any considerable size. Gradually the

gelatine on the surface becomes liquefied, and directly that this liquefaction has reached the sides of the test-tube, it begins to extend downwards. The whitish mass of the colonies now sinks to the bottom of the fluid gelatine, and as the liquefaction proceeds the colonies sink lower and lower, without the remnant of the whitish thread being altered in appearance. On agar-agar the comma bacillus forms whitish aggregations, which, however, are not characteristic. If sulphuric or hydrochloric acid be added to a cholera bacillus culture, a slight red coloration is produced. This, however, as has been recently discovered, is not peculiar to the cholera bacillus, but also occurs with the cultures of a large number of other bacteria, especially with those of some allied spirilla.

There are not a few organisms which are very similar to the cholera bacillus, both morphologically and in their development in cultures, and which have been frequently mistaken for it. Although they are not pathogenic in man, on account of this resemblance, a short description is here given of them.

Firstly, the *Spirillum Finckleri* presents morphologically an appearance not essentially different from that of the comma bacillus of Asiatic cholera. It may be a little thicker and plumper, but no distinction can be based on this. On this account at first they were confused with each other, and later the *Spirillum Finckleri* was considered to be the exciting

cause of cholera nostras, but it has since been discovered to be only a harmless inhabitant of the intestine, which multiplies with great rapidity during intestinal catarrh. It can easily be distinguished from the cholera bacillus by its growth upon gelatine, which is much more energetically liquefied by the former than by the latter. The colonies on gelatine plates can be seen with a low power to have quite clear sharp outlines, whilst no elevations or outgrowths are present; further, on the third day, they are twice as large as cholera colonies of the same age. Still more striking are the differences shown in puncture cultures in nutrient gelatine. No delicate whitish thread is developed along the track of the needle, but very soon a liquefaction funnel appears, which at first is uniform in width throughout its length, but which, during the course of development, becomes distinctly thicker in the upper than in the lower layers of the gelatine. Moreover, no bubble of air is ever formed at the mouth of the liquefaction funnel, whilst even by the third or fourth day the liquid gelatine has spread to the sides of the test-tube.

The cheese spirillum (*Spirillum tyrogenum*) comes between the cholera bacillus and the *Bacillus Finkleri*, both as regards their morphology and their behaviour in cultivations. They have been found in old cheese, and can be shown to be pathogenic in some animals. They may be easily distinguished from the

cholera bacillus by the more energetic manner in which they liquefy the gelatine. In this they resemble more closely the *Bacillus Finckleri*.

In South Russia, a bacterium, the *Spirillum Metschnikoffii* has been found in birds suffering from an intestinal disease; this morphologically, and in its behaviour in cultivations and towards staining reagents, so closely resembles the cholera bacillus that they cannot be distinguished one from the other. It is true that on gelatine plates a number of the colonies may conduct themselves in an anomalous manner, some looking very like Finckler's bacillus, whilst others cannot be distinguished from the cholera bacillus. But the only sure method of distinguishing the *Spirillum Metschnikoffii* from the cholera bacillus is by means of experiments upon animals. If, for example, a small quantity of the former is introduced with an inoculating needle into the breast muscle of a dove, the bird dies after twenty-four hours at longest of a specific disease, whereas it is almost completely unsusceptible to an inoculation of the cholera bacillus. An immense number of these spirilla are found in the tissue fluid of the affected œdematous muscle, and above all in the blood of the heart.

Another spirillum, which is pathogenic in man, is found in the blood of patients suffering from relapsing fever, an illness which is generally restricted to Eastern Europe and Asia, but which occasionally

occurs in the eastern provinces of Germany, and an epidemic which has been known in Berlin. This organism is very different in form from the preceding ones, resembling most the *Spirochæte plicatilis*, a very motile bacterium which is found in marshy water. In consequence of this resemblance, it has been called the *Spirochæte Obermeieri*. These spirilla form very thin, long threads with many convolutions, which are often bent upon themselves. They occur only in the blood of patients suffering from relapsing fever, and, strange to say, only during the onset of the fever. They are very motile, and retain this motility for some hours after leaving the human body. They have not been cultivated artificially; they can be easily stained with all the usual aniline dyes, but it is best to use methylene blue, though this reagent must however be used with care on account of the presence of the blood corpuscles in the cover-glass preparations. Monkeys are the only animals upon whom inoculation has had any effect.

CHAPTER XX.

ORGANISMS CAUSING PNEUMONIA AND DIPHTHERIA.

Various bacteria have been described as the exciting cause of pneumonia, especially of the croupous form. Either this illness may actually be caused by different organisms, or we are not in the position to say which is its real exciting cause in all cases. There are two bacteria, the *Bacillus pneumoniæ* and the *Micrococcus* or *Diplococcus pneumoniæ*, which occur most frequently, and which have been discovered in the rusty sputa of patients suffering from pneumonia, as well as in the tissues of those which have died from it. At present people are inclined to look upon the latter organism as the exciting cause of this disease. In isolated cases other organisms have been found, such as some of the bacteria of suppuration, but the *rôle* which these play in this disease is certainly only a subordinate one.

At first the *Bacillus pneumoniæ* was considered to be the sole excitant of the disease, but it appears that it is only rarely present in real pneumonia, although it has been frequently found in other illnesses of a

less severe nature. It grows on the ordinary nutrient media at blood heat as well as at ordinary room temperatures. In plate cultivations it develops in the form of white drops, which look like glistening knobs of porcelain; in stroke cultivations on agar-agar, a slimy greyish-white layer develops; in gelatine puncture cultures a fairly thick white thread is formed, whilst on the surface a similar knob is formed, which looks like the head of a nail, so that this culture has been called the nail culture. The bacilli are easily stained with the usual watery aniline dyes; they are also easily decolourised, and therefore cannot be treated according to Gram's method. They are small, short rodlets, generally occurring singly, but occasionally joined together in pairs. In the human body they are surrounded by delicate gelatinous sheaths, which in preparations of sputum or in sections of tissues do not become stained with the rest, but surround the rodlets like bright, strongly refracting zones. These sheaths or capsules are not seen in bacilli derived from cultures. This *Bacillus pneumoniæ* was considered by its discoverer, Friedländer, to be a micrococcus, and was called by him the Pneumococcus.

An organism, discovered by Fränkel, and now called *Diplococcus*, occurs much more frequently in croupous pneumonia. It is much smaller than the *Bacillus pneumoniæ*, occurs generally in pairs, and is

slightly pointed off towards its free ends. It is not merely a micrococcus, but a very short bacillus. Occasionally also little threads consisting of from four to eight cells are found. In the animal body this organism, like the pneumococcus, develops a fairly thick capsule, which never occurs in the artificially cultivated specimens. In the matter of staining, there is not much difference between this bacterium and the preceding one; but as it is not decolourised when treated according to Gram's method, they may be easily distinguished from one another by this means.

In cultures, Fränkel's *Diplococcus pneumoniæ* exhibits very marked peculiarities. It is very difficult to cultivate, and hence the greatest attention is necessary. First it requires a slightly alkaline medium; it is very susceptible to the least variation in the reaction of its medium. If this contains the smallest trace of free acid, the cultivation is a complete failure Further, the temperature must not sink below 24°; it grows most quickly and luxuriantly at a temperature of 37°. Hence it can only be cultivated on at least 15% gelatine, which must be kept at about 24°, since if only a slight rise of temperature occurs, the gelatine commences to liquefy. On gelatine plates very small transparent colonies with sharp outlines are formed; these only grow slowly, and never attain to any great size. The gelatine is never liquefied. In gelatine puncture cultures, colonies like those of

the *Streptococcus pyogenes* are formed, but are never so large, and are more transparent. Along the whole track of the needle there are a large number of small whitish spherical colonies, which do not coalesce. On agar-agar a very delicate shining transparent coating is formed, which also mainly consists of isolated colonies which, however, are confluent in a few places. This organism develops best in bouillon, which becomes very slightly turbid. It is remarkable for the exceedingly slight power of resistance of its cultures. They die off after a few days, and if their cultivation is to be continued, fresh inoculations must be made every three or four days.

It appears that this organism is not only the chief cause of pneumonia, but also of various other diseases. Moreover, it occurs very frequently in the mouth cavities of perfectly healthy men. As a rule, therefore, it appears to be a perfectly harmless inhabitant of the mucous membranes of the mouth and nose, and only under quite fixed, but at present unknown, conditions does it acquire the power of setting up disease. Apparently it is only able to spread when the resistant power of the animal cells has become weakened, as, for instance, after a chill, in consequence of which the mucous membrane becomes inflamed, and pours out fluid which affords a very favourable nutrient medium for the bacteria.

Fränkel's diplococcus is pathogenic in rabbits, in

guinea-pigs, and in mice; on the other hand, Friedländer's pneumococcus has only a slight effect on guinea-pigs, and none at all on rabbits.

The diphtheria bacillus is an organism similar in many respects to these. It occurs in the form of thick rodlets about ·004–·006 mm. in length, which may be either slightly curved or straight, and which have rounded off ends. Thus we see that it has no definite characteristic form. It is indeed remarkably polymorphous, being sometimes thickened at one pole, and sometimes at both, when it looks something like a dumb-bell. Occasionally, in long bacilli, a third swelling occurs in the middle, or, as in the tubercle bacilli, one or more gaps are present, so that it appears as though the organism were broken up into several pieces. As a rule it has no gelatinous sheath, but sometimes it happens that it is surrounded by a colourless, not especially wide capsule. Generally the bacilli are isolated, but occasionally a few cells collect together to form clumps.

It is a very difficult matter to stain these bacilli; since, if the ordinary aqueous solutions are used, as a rule, only quite unsatisfactory results are produced; only certain parts, generally the poles, take up the stain well, whilst the interlying portions are either colourless or only very slightly coloured. Experiments with Löffler's methylene blue are somewhat more successful, especially if we have to deal with young cul-

tures, or if we wish to demonstrate the presence of the bacilli in false membranes. Gram's method is quite useless for the diphtheria bacillus, for it either becomes quite decolourised, or only a very small portion in the interior of the cell remains faintly stained. Even the other staining solutions are not suitable, for the bacilli, already so varying in their shape, become still more changed by powerful staining reagents.

These bacilli only develop at a temperature of over 20°. If the same gelatine is used as for the *Diplococcus pneumoniæ*, there appear on the culture plates small disc-shaped, whitish colonies, which are seen with the microscope to have slightly jagged edges. Upon agar-agar cultivations kept at blood heat thin transparent coatings are at first formed; these, to begin with, only grow very slowly, but gradually they develop more rapidly as the bacteria evidently grow more accustomed to their soil. In agar-agar plate cultivations, which have been kept at blood heat for two days large colonies appear, which are about the size of pins'-heads; they are flat and lenticular in shape, and of a greyish white colour; when they reach the surface they are very glistening and more transparent. If such a colony is examined with a low power, a very characteristic appearance may be observed. The small colonies with their irregular outlines look like an innumerable quantity of rough granules packed closely together. Amongst the better

known kinds of bacteria this appearance is only to be seen in the *Bacillus megaterium*. But from this the diphtheria bacillus can be very easily distinguished, both by its form, and by the fact, that it never liquefies the gelatine, whereas the *Bacillus megaterium* gradually does so. In gelatine puncture cultivations a growth appears, which is similar to that of the *Streptococcus erysipelatos*; a great number of small round whitish colonies develop along the track of the needle; on blood serum there forms a somewhat thick whitish coating which is quite opaque, at any rate in the middle. But the most characteristic of all is the culture in bouillon. Here small solid granules of a whitish colour are formed, which either sink to the bottom, or fasten themselves upon the sides of the vessel. As a rule the bouillon does not become at all turbid, but occasionally a slight cloudiness occurs.

The diphtheria bacillus is to be found chiefly in the diphtheritic membrane, especially in the older portions; it has however been found in healthy men. It may be that it resembles the Fränkel's diplococcus in this, but it is also possible that, in these isolated cases, some of the organisms present in the mouth cavity, which are very similar to the diphtheria bacillus, may have been mistaken for it.

CHAPTER XXI.

BACTERIA PATHOGENIC IN ANIMALS.

Amongst the kinds of bacteria described in the preceding chapters, there are a great many which are also pathogenic in animals, and amongst these some which only comparatively rarely occur in man. However, since they frequently cause more or less severe diseases in him, they have for us a special interest, and hence have been described in some detail. But of the bacteria mentioned in this chapter, so far as is known at present, one only, the glanders bacillus, is also pathogenic in man, whilst the others are pathogenic in a greater or less number of animals. Of these, in consequence, only a short account will be given.

The glanders bacillus occasions, especially in horses and in similiar animals, a very violent and infectious disease, called glanders or farcy. The bacilli are motionless, exceedingly delicate and short, with their ends rounded off; they occur either singly or joined together in pairs. Apparently they form spores, since small strongly refractive granules are to be seen in the cells; these granules have quite lately been distinguished from the surrounding plasma of the bac-

teria cells by means of a method of contrast staining; hence in this respect they behave like spores. Still their germination has not yet been observed, on which account their nature cannot be definitely determined.

The glanders bacilli cannot very well be cultivated on gelatine, for they can only grow at a temperature at which it begins to be liquefied. Nutrient agar-agar must therefore be used, to which 3 to 4 per cent. of glycerine may with advantage be added. If the agar-agar plates are kept for two days in the incubator at a temperature of 37°, the colonies are distinctly to be seen. Small, round, dirty-white flakes are formed, which are distinctly translucent at the edges, and quite bright on the surface. With a low power the colonies are seen to have sharp edges and a very slightly granular surface. On stroke cultivations in glycerine agar-agar, a dirty white, slimy, translucent coating is formed. The culture on blood serum is almost transparent and slightly yellow in colour; generally round the edges of the stroke culture in this medium a few free colonies occur, which either remain permanently isolated, or coalesce at a few places on the circumference. The growth of the glanders bacillus upon potatoes is especially characteristic; if the preparation is kept at blood heat, a very luxurious growth takes place. Along the inoculation stroke a slimy coating appears, which at first is yellowish and almost transparent, but which becomes

gradually darker and darker until finally after eight days it has assumed a rather intense rusty red colour. No other known species of bacterium grows in a similar manner upon potatoes, and therefore the glanders bacillus may be invariably recognised by its behaviour in these cultures.

The glanders bacillus may be stained very readily by any of the ordinary aqueous staining solutions: it is also very easily decolourised even by the weakest reagents. On this account Gram's method cannot be employed, nor can a contrast staining be accomplished.[1] A good method is to treat the cover-glass with warm carbolic fuchsine or carbolic methylene blue, and then to rinse it either with pure distilled water or with water containing at the very most $\frac{1}{10}$ % of hydrochloric acid.

As the glanders bacillus is pathogenic in man, it is necessary to take the greatest care in performing all experiments with it. By degrees the bacilli lose their virulence, after having been repeatedly inoculated into artificial media.

Swine erysipelas, a disease very prevalent in Germany, and also in some districts of other European countries, and which is often very virulent, is caused by very small slender bacilli, which are found in exceedingly great numbers in the bodies of affected animals which have died of the disease, especially in the capil-

[1] See note on p. 212.

laries of the internal organs. They occur, as a rule, either singly or in pairs; occasionally, however, they are found in long threads, which are then often matted; they are motile, and grow as well at ordinary room temperatures as at blood heat. On gelatine plate cultivations, after a few days, peculiar bluish-grey opalescent films are formed; these rarely appear on the surface of the plate, but seem to prefer to spread themselves out on the surface of the glass. These films can be best distinguished if the plate is placed on a piece of black paper. It can then be seen with a magnifying glass that the film consists of innumerable very delicate tangled thread-like strands, which are almost transparent, and only to be distinguished from the background in consequence of their opalescence. These strands consist of bacterium threads which have grown together.

A similarly peculiar growth occurs in a puncture cultivation; in this case bluish-grey cloudy bundles are formed, which stretch out at right angles in all directions from the line of inoculation, and which appear to be piled up one above the other, so that the culture has been called the test-tube brush culture, from its resemblance to the brush. Such a cultivation cannot be confused with any other except that of the *Bacillus murisepticus*. On agar-agar this swine erysipelas bacillus develops very small, hardly visible transparent droplets; in bouillon thick heaps of bac-

teria are formed. It does not grow upon potatoes. Apparently it possesses the power of spontaneous movement; and the formation of spores has already been observed in it. It can be stained extremely easily with any of the ordinary aqueous aniline solutions, but the preparations succeed especially well if treated according to Gram's method, which may also be employed for staining sections containing these bacteria.

Apparently the swine erysipelas bacillus is identical with the mouse septicæmia bacillus (*Bacillus murisepticus*). The slight differences which are perceptible in their cultures in gelatine may probably be accounted for by the different conditions under which they have been placed in their respective hosts. The *Bacillus murisepticus* is somewhat the more slender of the two, and in gelatine puncture cultivations the cloudiness spreads out further, nearly reaching to the sides of the glass. When various different animals are inoculated with it, they are affected just as if they had been inoculated with the swine erysipelas bacillus.

A very virulent epidemic occurs amongst fowls, the chicken cholera, or fowl typhoid. It is caused by short, thick, almost ellipsoid bacilli, with rounded off ends; they occur as a rule either singly or in pairs, but are occasionally united together in chains. Their cultivation is easy, but no characteristic peculiarities are to be observed. Small whitish punctiform colonies

are formed after a few days on plate cultivations; in puncture cultures thin white granular threads develop; in stroke cultivations on gelatine a very tough grayish-white coating is formed, which clings very closely to the gelatine, and is not very bright, whereas the stroke cultures on agar-agar are very bright in appearance.

Their staining is not difficult, but they are very easily decolourised, on which account Gram's method cannot be employed. Aqueous solution of methylene blue is very suitable for this purpose, and Löffler's methylene blue is also to be recommended. A quite unique peculiarity is to be perceived in stained preparations; the greater number of the individual cells are not evenly stained; only the two poles of the very short rodlet are coloured, whilst a narrow space in the middle remains unaffected. At first it looks as though we were dealing with a diplococcus, but careful observation shows us that this is not the case, for the cell membrane, even of the unstained portions, can be clearly distinguished.

A virulent epidemic occurring amongst wild animals, called the game plague, is caused by a pathogenic organism which is very similar to the bacillus of fowl cholera; in a similar manner bacilli are found in ferret plague, rabbit septicæmia, duck cholera, swine plague, pig typhoid, and hog cholera, which are either quite similar to the chicken cholera bacillus, or hardly to be distinguished from it. Some of these

are certainly identical with it, and others very closely connected.

An epidemic sometimes occurs amongst cattle, which shows itself in the formation of swellings in the region of the jaws. These swellings contain sometimes thick, sometimes thin pus, which contains hard yellow granules. These yellow granules are seen with the microscope to be of a raylike structure, and consist of an organism which is at present perhaps mistakenly classed with bacteria, and which on account of its form is called the ray fungus—*actinomyces bovis* or *hominis*. Generally the granules are composed of a number of clublike threads which are swollen up into knobs and amongst which more threads, resembling bacteria or even chains of cocci are to be found. Whether these last really belong to the actinomyces or not rather to other organisms which have been introduced at the same time into the swelling, is at present not yet determined. The ray fungus has been observed in men, causing a disease which not infrequently proves fatal. It is not easy to cultivate, as it only grows out of contact with air; the appearance it presents in cultures is somewhat different from that described above.

Staining is most successfully performed by means of Gram's method, and only when they are stained can the true nature of these granules be distinguished. A similar but somewhat different organism appears in the striated muscles of the pig.

EXPLANATION OF THE PLATES.

Fig. 1 represents Staphylococcus pyogenes citreus, from an agar-agar culture; in addition to single cells, diplococci, tetrads, and short threads consisting of three cells hanging together, are seen. Stained with aqueous solution of fuchsine.

Fig. 2. Micrococcus gonorrhœæ stained with methylene blue See text.

Fig. 3. Streptococcus erysipelatos, stained for 24 hours with very dilute aqueous gentian-violet solution.

Fig. 4. Threads of the anthrax-bacillus, with spores. Stained with hot aniline water fuchsine, decolourised with sulphuric acid, so that only the spores remain stained.

Fig. 5. Bacillus tuberculosis, preparation has been made from sputum very rich in tubercle bacilli, stained with hot aniline water fuchsine, decolourised with nitric acid, and not re-stained, so that only the bacilli are to be seen in the photograph.

Fig. 6. Cholera bacilli from a somewhat old cultivation, on which account the individual cells are not uniform. Stained for twenty-four hours with acqueous carbolic fuchsine solution.

Fig. 7. Typhoid bacilli from an agar-agar cultivation, stained for twenty-four hours with aqueous gentian violet.

Fig. 8. Typhoid bacilli with flagella, stained according to Löffler's method.

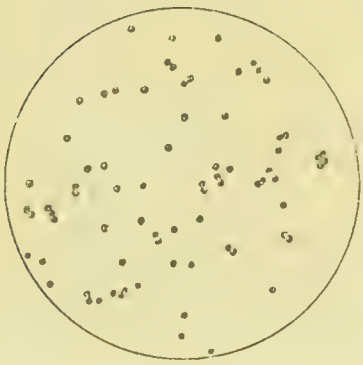

FIG. 1.

Staphylococcus Pyogenes Citreus.
× 1000.

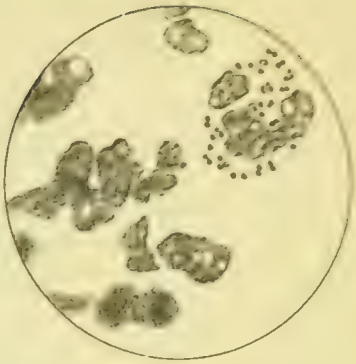

FIG. 2.

Micrococcus Gonorrhœæ.
× 1000.

FIG. 3.

Streptococcus Erysipelatos.
× 1000.

FIG. 4.

Anthrax Bacillus with Spores.
× 1000.

FIG. 5.

Bacillus Tuberculosis.
× 1000.

FIG. 6.

Spirillum Choleræ Asiaticæ.
× 1000.

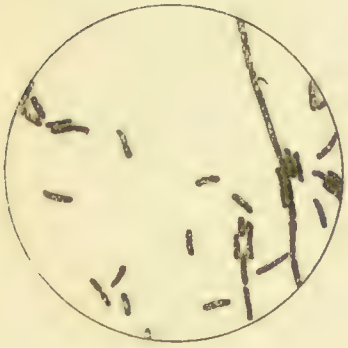

FIG. 7.

Typhoid Bacillus.
× 1000.

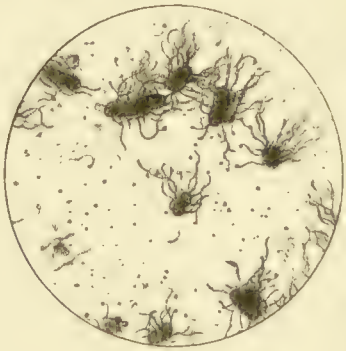

FIG. 8.

Typhoid Bacillus with stained Flagella.
× 1000.

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Butler & Tanner, The Selwood Printing Works, Frome, and London.